Microbial populations associated with the Irish brown seaweeds, *Ascophyllum nodosum* and *Laminaria digitata*, and their biotechnological applications.

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Declaration of independence

I hereby declare that this thesis and the work presented within is my own work and has not been submitted for another degree at University College Cork or elsewhere.

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Wada Maureen Ihua

"*The Spirit of The LORD GOD is upon me. His breath has given me life. I am able to do all that I have been called to do. I never lack Grace. I am never disadvantaged. I am a light to my world, always and forever*"

Wada Maureen Ihua

(*Sunshine*)

.

This thesis is dedicated to all the teenagers at RCCG Inspiration House, Cork (*my kids*) and to my unborn children. For you too, all things are possible with Christ.

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Abstract

Brown macroalgae or seaweeds which belong to the class Phaeophyta arose around 200 million years ago and have since become one of the most ecologically remarkable and commercially relevant marine species. Brown seaweeds such as *Ascophyllum nodosum* and *Laminaria digitata* are a rich source of polysaccharides including fucoidan, alginate, laminarin and ascophyllan which have a variety of functional biotechnological applications. These seaweeds are well known to harbour a diverse range of bacteria which exert both beneficial and detrimental effects on their algal host. Some of the positive ecological functions of the associated microbiome include modulating the normal morphology of their host as well as contributing to antifouling defence. Marine microorganisms associated with algae are also known to produce diverse hydrolytic enzymes such as polysaccharidases which improve algal nutrient uptake and development while also contributing to algal defence from potential grazers. These diverse and complex bacterial communities thus represent a potential source of novel enzymes with biotechnological applications that could include their use in enzyme-assisted extraction (EAE) strategies, resulting in the improvement of the yields of algal components with cosmeceutical, functional food, and nutraceutical and biopharmaceutical applications. Several environmental and non-environmental factors, including seasonal variations and the physiological (fresh or decayed) state of macroalgae, can influence the composition and abundance of algal-associated epibacterial communities. The original work presented in this study aims at describing the microbial populations that are associated with the two brown seaweeds, namely *Ascophyllum nodosum* and *Laminaria digitata* and to examine the hydrolytic potential of their cultivable surface microbiota which may display useful biotechnological applications.

Chapter 1 presents an overview of macroalgae and macroalgal-microbial interactions and of the diversity of algal-associated microbial communities and key interactions with the algal host.

In addition, it describes the complex nature of brown algal cell walls and the potential for their exploitation. **Chapter 2** describes the use of both metagenomic and culture-based approaches to investigate the effect of algal decay on the composition and abundance of *Ascophyllum nodosum* associated bacterial population. Three batches of fresh *Ascophyllum nodosum* samples were incubated in artificial seawater under sterile conditions in the laboratory for a period of six weeks, each at a different temperature (18 °C, 25 °C and 30 °C) to induce decay. Over 800 bacterial isolates were cultured from the intact and decaying macroalga using both traditional cultivation methods including the novel ichip *in-situ* cultivation method. Distinct differences between the bacterial populations associated with the seaweed before and during decay were observed. We also identified over 50 bacterial isolates which are capable of producing algal cell wall polysaccharide degrading enzymes of interest, some of which were tested for their biotechnological application and were proven to be successful, with 11-13% yield in the enzyme-assisted extraction of phenolics from a brown seaweed. Similarly, using culture dependent and independent methods, **Chapter 3** describes how the induced decay of the brown seaweed *Laminaria digitata* resulted in changes in its associated microbial communities. We incubated the seaweed in the laboratory for four weeks at 20 °C, 25 °C and 30 °C. Our results show that the cultivable surface microbiota of the macroalga is enriched with algal cell wall degradation enzymes, with over 50% of the bacterial isolates being found to produce at least one of the enzymes tested.

The study presented in **chapter 4** further describes how morphological niches and seasonal variations drive the structure and composition of *Laminaria digitata* associated bacterial communities. Using Illumina sequencing of the 16S rRNA genes, the metagenomic populations of the four different parts (holdfast, stipe, meristem, blades) of the seaweed were examined over a ten month period, with samples being collected in April 2016, July 2016, November 2016 and January 2017. The data obtained revealed that the seaweed appears to lack

a core microbial community, and that the month of November and the holdfast region harbour the most diverse bacterial population.

Finally, the study ends with **chapter 5** with a general discussion of the relevance of the results obtained and a look at some possible future prospects.

Chapter 1: General Introduction

1.1 Brown algal Taxonomy and Morphology

The infrakingdom Heterokonta (also known as Stramenopiles or heterokonts) comprises of photosynthetic organisms possessing flagella with tripartite tubular hairs, including golden algae (Chrysophyta), brown algae (Phaeophyta) and diatoms (Bacillariophyta), as well as other non-photosynthetic organisms with lost or reduced tripartite hairs, such as water moulds (Andersen, 2004). Heterokonts are believed to have descended from an endosymbiotic event between a unicellular red alga and a non-photosynthetic protist (Reyes-Prieto et al., 2007) and the earliest fossil studies suggest that photosynthetic heterokonts arose about 1000 million years ago (Brown and Sorhannus, 2010). The appearance of the Phaeophyta class or brown algae is believed to have occurred 200-300 million years ago, in an evolution event which offered multicellularity and plant-like structures (Brown and Sorhannus, 2010; Charrier et al., 2018; Silberfeld et al., 2010).

Some of the earliest taxonomic identification of brown algae were derived from the physiological and anatomical features of the marine group, including the morphology of the thallus, life cycle, growth mode and reproductive style (Papenfuss, 1951; Rousseau and De Reviers, 1999; Wynne and Loiseaux, 1976). The advent of a number of improved molecular techniques within the last two decades have helped provide better insights into the taxonomy and phylogeny of brown algae (Phillips et al., 2008; Silberfeld et al., 2010; Silberfeld et al., 2011). To date, there are more than 250 known genera (De Reviers et al., 2007) within the brown algal class, with 1500-2000 species being identified (Silberfeld et al., 2010). The majority of brown algae exist in marine environments, with only less than 1 % of the species being found in freshwater habitats (De Reviers et al., 2007).

These photoautotrophic multicellular seaweeds are characterized by a distinct golden brown colour which arises from the presence of a number of carotenoid pigments, including fucoxanthin, violaxanthin and chlorophylls a, c1 and c2 in their plastids (De Reviers et al., 2007). While Phaeophyta share common features such as plastids and lamellae, which are characterized by trio-stacked thylakoids, as well as a girdle lamella with their ancestors (Wehr, 2003); they possess distinct evolutionary autapomorphies. For example, the protoplasmic connections between adjacent cells, also known as plasmodesmata, found in these macroscopic marine organisms are self-developed (Raven, 2018) but lack desmotubules, whereas this feature is present in green algae (Terauchi et al., 2015). Seaweeds grow to a variety of sizes, ranging from a few centimetres to up to 45 m in length, for example, *Macrocystis pyrifera* which forms giant kelp forests (Schiel and Foster, 2015). Furthermore, brown algal thalli display a varied number of vegetative organization. While some members are organized in branched filaments which lack a heterotrichous arrangement, others such as those in the order *Laminariales* are rather complex, with sieve tubes developed for photosynthesis (Lobban and Wynne, 1981).

1.1.1 Ascophyllum nodosum

Ascophyllum nodosum (Figure 1.1) belongs to the family *Fucaceae* and is currently the only species in the genus *Ascophyllum* (Kadam et al., 2015). It is common in sheltered to moderately exposed marine geographical sites and is distributed along the North-Eastern coast of North America and the North-Western coasts of Europe, being reported in Ireland (2019), Norway (Round, 1981), Netherlands (Stegenga et al., 1997), Britain and Isle of Man (Hardy et al., 2006).

Ascophyllum nodosum is attached to rocks by means of its holdfast and can grow up to 2 m long, with up to 16 cm per year growth year being recorded, and has an extended life span of 10-20 years (Stengel and Dring, 1997). The long olive-green to olive-brown coloured fronds

are tough and leathery, possessing an irregular dichotomous branching, and lack a midrib (Hiscock, 1979; Newton, 1931; StP et al., 2017). It is also known for its distinct egg-shaped single bladders which are finely arranged along its fronds and are responsible for the common alternative name for this brown seaweed, egg wrack.

Figure 1.1: *Ascophyllum nodosum* (left) and *Laminaria digitata* (right)

1.1.2 Laminaria digitata

Laminaria digitata (Figure 1.1), also known as oarweed, is a large brown seaweed which belongs to the family *Laminariaceae*. This perennial alga is distributed along the lower intertidal and shallow subtidal zone of the coasts of Britain and Ireland, with the exception of the east and south-east coast of England. It is also common in Svalbard, Northern Russia, Iceland and France (Hill, 2008).

The morphology of oarweed is distinct from that of other similar brown seaweeds. It is dark brown in colour and possesses a tough leathery texture. The holdfast firmly anchors it to rock sediments with rhizoid-like protrusions. Its stipe which grows up to 5 ft in height has an oval cross section and is smooth and flexible – a feature which offers it resilience against seawater

currents. In addition, the laminate blade of *L. digitata* can reach up to 1.5 m in length and splits into a number of finger-like segments (Guiry, 2019).

1.2 Macroalgal-microbial interactions

1.2.1 Diversity of algal-associated microbial communities

Surface colonization is ubiquitous in marine organisms and marine macroalgae are no exception. In particular, macroalgal surfaces provide a suitable water-solid interface for the growth and colonization of diverse bacterial communities. The presence of carbon rich constituents in macroalgal cell walls such as agar, carrageenan, alginate, cellulose and pectin (Egan et al., 2013) together with other organic algal exudates is likely to be a factor in helping to contribute to bacterial colonisation of algal surfaces, as these constitute a rich nutrient source that supports the growth and proliferation of epibacterial communities. Cundell and co-authors (Cundell et al., 1977) report one of the earliest studies on the density of *A. nodosum* surface-attached bacteria using scanning electron microscopy. Dense lawns of bacteria were found on the holdfast and intermodal segments of the algal thallus, with fewer cell counts being observed around the apical tips.

Martin and co-workers (Martin et al., 2015) report that the cultivable surface of *A. nodosum* is comprised of a diverse range of bacteria belonging to up to 36 different genera including *Cobetia, Marinomonas, Pseudoalteromonas* and *Cellulophaga*. Likewise, molecular studies on *Ulva australis, Laminaria hyperborea, Nereocystis luetkeana* and *Macrocycstis pyrifera* have also revealed the presence of a wide range of diverse bacteria (Bengtsson et al., 2012; Burke et al., 2011b; Weigel and Pfister, 2019). The majority of these seaweed-associated bacteria are taxonomically identified as belonging to the phyla *Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria* and *Planctomycetes.* In addition, seaweed-associated epiphytic bacterial communities have been reported to differ distinctively from the surrounding seawater bacterial populations (Burke et al., 2011b; Staufenberger et al., 2008). This further demonstrates the

uniqueness of the macroalgal surfaces as a suitable host for the proliferation of selected bacterial species.

1.2.2 Variations in the structure of algal-associated microbial communities

The assembly and maintenance of bacterial populations on algal surfaces is well known to be modulated by a number of physical, chemical and biological factors, including the nature of the algal host (Staufenberger et al., 2008) as demonstrated in Figure 1.2 (Morrissey et al., 2019). Previous molecular studies which demonstrate differences between the associated microbiome of four algal species (*Delesseria sanguinea*, *Fucus vesiculosus*, *Saccharina latissima* and *Ulva compressa*) (Lachnit et al., 2009) indicate that the recruitment of bacterial species can be host-specific. (Weigel and Pfister, 2019) also reported significant differences between the epibacterial communities associated with the canopy-forming kelps, *Macrocystis pyrifera* and *Nereocystis luetkeana*, in particular; with a greater relative abundance of *Verrucomicrobia* being identified on the latter seaweed. The microbial symbiont community of *M. pyrifera* was found to be more diverse with a mean ASV richness per sample of 102 whereas *N. luetkeana* communities hosted fewer (mean ASV richness per sample of 36) dominant epibionts. The associated microbial community profile of algal species may differ according to the composition and abundance of the algal metabolome being produced which can either attract or repel certain bacterial groups (Potin et al., 2002). Some brown seaweeds for example, exude high concentrations of mannitol (Gravot et al., 2010) which has been demonstrated to influence biofilm formation in *Pseudoalteromonas* sp. and *Zobellia galactanivorans* (Salaün et al., 2012). Metabolic cross-exchange between microbes and their algal host is crucial in establishing and maintaining the community structure (Mee et al., 2014), with different bacteria contributing specific ecological functions. However, studies indicate that different bacterial species can perform similar ecological functions, thus allowing for the

existence of generalist epiphytes which may be common in different algal species (Burke et al., 2011a; Burke et al., 2011b; Morrissey et al., 2019).

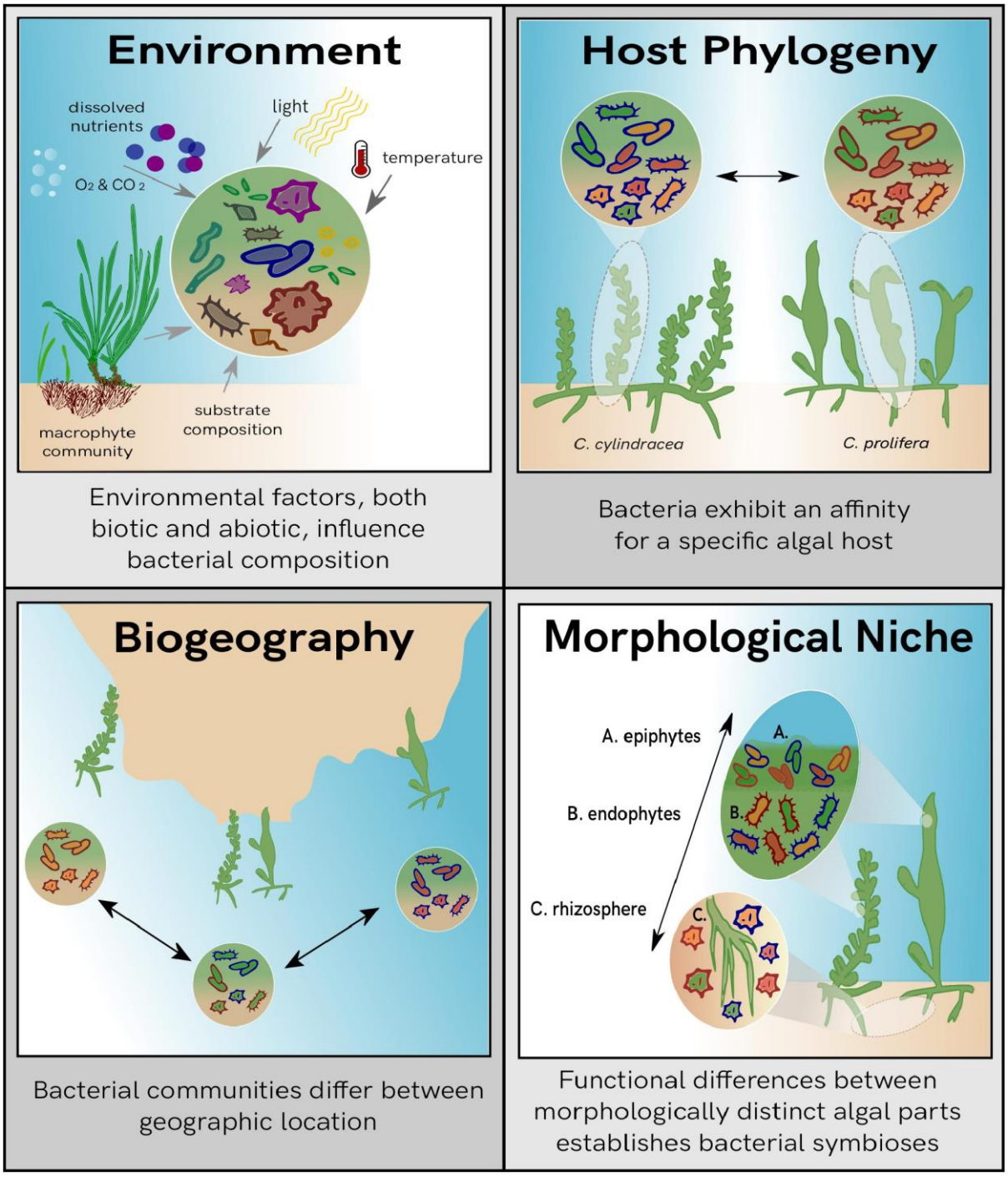

Figure 1.2: Potential biological and non-biological factors which influence algal bacterial populations. Adapted from (Morrissey et al., 2019).

Variations between morphological niches within an individual algal thallus part has also been observed. Within *Nereocystis luetkeana*, distinct microbial community differences have been identified between the microbial communities associated with the meristem and the apical blade regions. While the meristem displayed low total bacterial abundance and evenness, the algal blade tip was found to be more developed and complex (Weigel and Pfister, 2019). Transcriptomic analysis also identified differences between the bacterial communities associated with basal and apical tissues of the green alga, *Caulerpa taxifolia* (Ranjan et al., 2015). Epibacterial communities of root-like regions of *Caulerpa* species have been linked to distinct metabolic roles such as nitrogen fixation, which is consistent with the specialized nutrient uptake and substrate adhesion function of the holdfast. In another study (Staufenberger et al., 2008), the young meristem and cauloid tissues of *Saccharina latissima* were found to be more closely related, with respect to their microbiota; whereas the rhizoid and phyloid tissues formed distinct bacterial communities.

Further investigations have reported that variations in bacterial community structures derived from the same algal species may also arise due to fluctuations in environment parameters, together with differences in geographical locations (Figure 1.2), as seen in *Cystoseira compressa* (Mancuso et al., 2016), *Saccharina latissima* (Staufenberger et al., 2008), *Laminaria hyperborea* (Bengtsson et al., 2010), *Macrocystis pyrifera* and *Nereocystis luetkeana* (Weigel and Pfister, 2019). Geographical locations as well as seasons are characterized by distinct environmental markers such as salinity, nutrient availability and temperature, which combine to create a unique "biogeographic signature" for bacterial colonization (Morrissey et al., 2019). Variations in this biogeographic signature are thus likely to result in temporal variations in the related bacterial populations.

Beyond the species type and environmental fluctuations, algal-associated microbial assemblages can also be described according to the developmental stage and life history of the

alga. The different physicochemical properties of the algal surface at different vegetative stages may create niches which are suitable for colonization by distinct epiphytic bacteria. Perennial seaweeds are likely to harbour more advanced and diverse microbial communities when compared to annual macroalgae, as observed in the associated microbiome of *N. luetkeana* and *M. pyrifera* (Weigel and Pfister, 2019). Some authors propose that perennial species, some of which are characterized by the growth and senescence of blade tissues in summer and autumn respectively, serve as a reservoir for the bacterial colonization of new tissues (Bengtsson et al., 2010; Lemay et al., 2018). Similarly, the age of the algal thallus was considered as an explanation for the lower abundance of *Planctomycetes* being observed in *Laminaria hyperobrea* samples collected in February when the kelp lamina is older, than in July and September (Bengtsson and Øvreås, 2010).

1.2.3 Key interactions of macroalgal-associated microbial communities

While seaweeds primarily provide microbes with carbon-rich nutrients for growth, epiphytic bacteria also play critical roles in the establishment and development of the normal morphology of their algal host. Several authors have reported studies which provide some insights on key seaweed-microbial interactions which will be further discussed.

1.2.3.1 Nutrition and Growth

Metagenomics and functional genomic analyses of algal associated bacterial communities have revealed that in addition to providing carbon dioxide to support photo-autotrophism in their algal host, epiphytic bacteria possess genes encoding proteins which are responsible for the utilization of phosphates, nitrates and a number of other growth substances (Egan et al., 2013; Singh and Reddy, 2014). Recent investigations into the associated microbiome of the red alga *Laurencia dendroidea* also identified gene clusters related to carbohydrate and amino acid metabolism (de Oliveira et al., 2012). While bacteria utilize polysaccharide and lipid sources from their algal host for energy, relevant processes involved in nitrogen fixation which are beneficial to the host also appear to occur. *Cyanobacteria* and other nitrogen-fixing bacterial

species have frequently been reported to be present on numerous macroalgae including *Cladophora* (Byappanahalli et al., 2019) and *Codium* (Chisholm et al., 1996) species. The nitrogen fixation capabilities of algal associated bacteria contributes to the host algal growth and to its development.

1.2.3.2 Normal morphology

Epiphytic bacteria have a marked effect on the normal development of several algal species. For example, axenic plantlets of the green alga *Ulva linza* lack a hollow tubular unicellular thallus and aberrantly develop into undifferentiated cells, whereas, in the presence of bacterial colonization, several monostromic tubular structures are formed (Marshall et al., 2006). Similar effects have also been reported for other *Ulva* species including *Ulva fasciata* (Singh et al., 2011), *Ulva intestinalis* (Ghaderiardakani et al., 2017) and *Ulva mutabilis* (Ghaderiardakani et al., 2017), as well as *Ectocarpus* sp. (Tapia et al., 2016) and *Monostroma oxyspermum* (Matsuo et al., 2003). The mechanism through which epiphytic bacteria modulate the normal morphogenic development of macroalgal species is currently not well understood. However, there have been reports on the possibility of bacterial metabolites being responsible for this physiological effect. One such metabolite is thallusin, which was first isolated from the bacterial strain YM2-23 found on *Monostroma oxyspermum (Matsuo et al., 2003; Matsuo et al., 2005)*. The gradual reversal of normal thallus morphology upon deprivation of thallusin suggested that macroalgae may depend on a continuous supply of the potent inducer to maintain their algal structures. Other compounds similar to plant cytokinins and auxins which are produced by macroalgal epiphytic bacteria (members of the genera *Sulfitobacter*, *Roseobacter*, *Halomonas* and *Maribacter*) have also been reported to contribute to growth and differentiation in *Ulva mutabilis* (Spoerner et al., 2012).

1.2.3.3 Enhancement of zoospores

The adherence and settlement of zoospores on a suitable substratum which supports germination is a critical event in the life cycle of most algal species (Singh and Reddy, 2014).

Studies demonstrate that zoospores adhere to microbial biofilms on the surfaces of seaweeds and that certain microbial interactions can in fact increase the number of algal zoospores (Joint et al., 2000; Thomas and Allsopp, 1983). Joint et al. (2000) reports a preferential selection of certain bacterial strains within a surface biofilm for the attachment of *Entermorpha* zoospores and demonstrated that both the density and age of the bacterial biofilm (Shin, 2008) can significantly contribute to the settlement of the algal spores. Macroalgal zoospore settlement has also been shown to be influenced by physical and chemical properties of microbial biofilms including wettability, surface energy, surface chemistry, adhesion and topography (Singh and Reddy, 2014)

1.2.3.4 Antifouling defence

While it is true that macroalgal surfaces are attractive for microbial colonization, biofouling, an undesirable attachment of unwanted microbes and sessile invertebrates, can pose serious problems to the host algal species. Overall, marine biofouling alters the dynamics of species interaction and affects biodiversity as alien species are introduced (Carvalho et al., 2017). In shellfish aquaculture, for example, biofouling control is estimated to cost up to US$ 3 billion per annum (Fitridge et al., 2012). It is not surprising therefore that numerous studies demonstrate that macroalgae, both independently and in association with their epiphytic bacteria, produce substances which possess inhibitory actions against fouling organisms (Dahms and Dobretsov, 2017; Engel et al., 2006; Oguri et al., 2017; Schwartz et al., 2017).

For example, hydrophilic and lipophilic extracts from over 40 algal species have been reported to possess potent antimicrobial activities against pathogenic microbes including *Dendryphiella salina, Pseudoaltermonas bacteriolytica* and *Lindra thalassiae (Engel et al., 2006). The red alga Delisea pulchra* on the other hand secretes halogenated furanones which inhibit the growth and attachment of a number of bacterial species. Furanones are released from the central vesicle of gland cells onto the algal surface and inhibit *N*-acyl-L-homoserine lactones (AHL)-

dependent gene expression which is critical to bacterial quorum sensing. The concentration of furanone on different sections of *D. pulchra* thallus has been correlated with bacterial abundance and further electron microscopy revealed lower bacterial density on the red alga when compared to other algal species which do not produce furanones (Dworjanyn et al., 1999; Maximilien et al., 1998). In addition, novel biogenic compounds including omaezol, hachijojimallenes A and debromoaplysinal, recently isolated from *Laurencia* sp. have been demonstrated to display potent antifouling activities against barnacle larvae and diatoms (Oguri et al., 2017).

Epiphytic bacteria isolated from seaweeds also contribute to the algal host defence against biofouling organisms as well as unwanted colonization. One mechanism common to most algal associated microbes is the production of antimicrobial compounds. The cultivable surface microbiota of *Saccharina latissima* is known to be enriched with up to 100 strains with antimicrobial activity, across a range of diverse taxonomic groups (Wiese et al., 2009) while *Asparagopsis armata*-associated bacterial populations have also recently been reported to display antibacterial activities against a wide range of bacteria (Horta et al., 2019). In particular, extracts from *Pseudoalteromonas* species have been shown to inhibit the attachment and growth of fouling organisms including benthic diatoms, fungi and bacteria, algal spores and invertebrate larvae (Bowman, 2007; Egan et al., 2001; Egan et al., 2008; Rao et al., 2007).

1.3 Biotechnological relevance of macroalgae and macroalgal-associated bacteria

1.3.1 Medical and pharmaceutical applications

Owing to their biochemical properties, seaweeds and seaweed-associated bacteria are widely used in a variety of medical and pharmaceutical applications which shall be discussed in this section.

1.3.1.1 Medicinal

The beneficial medicinal effects of the consumption of seaweed are quite well established. In a survey of over 3,000 participants in Korea, Lee and co-workers (Lee et al., 2010) suggested that the consumption of edible seaweed may decrease the risk of type II diabetes. Consumption of a commercially available blend of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus* was also found to be associated with up to 7% significant increase in insulin sensitivity (Paradis et al., 2011). In other studies, the concentration of postprandial glucose was reduced when carbohydrate diets were supplemented with the seaweed Wakame (*Undaria pinnatifida*) and Mekabu (sporophylls of Wakame) (Tanemura et al., 2014), and the levels of triglycerides decreased upon the consumption of up to 48 g of seaweed per day for four weeks (Kim et al., 2008). Edible seaweeds have also been linked with raising the levels of serum thyrotropin (TSH) and iodine, involved in regulating thyroid disorders (Combet et al., 2014; Miyai et al., 2008; Teas et al., 2007).

1.3.1.2 Antiviral

One of the most studied algal polysaccharides with respect to antiviral properties is carrageenan, which is abundant in red seaweeds. Leibbrandt and co-authors (Leibbrandt et al., 2010) report the effective treatment of influenza virus-infected mice with a nasal spray containing iota-carrageenan. Human nasal epithelial cells treated with the sulfated polysaccharide, carrageenan, survived virus-induced cell death more than cells which were treated with a control polymer, CMC. While up to a 50% decrease in the cytopathic effect of influenza virus on Madin-Darby Canine Kidney (MDCK) cells treated with carrageenan has also been reported (Leibbrandt et al., 2010). Furthermore, carrageenan elicited anti-viral activities have been reported which are comparable to the commercially available neuraminidase inhibitor drug, oseltamivir; and it has been proposed as a potent treatment alternative, particularly in light of the emergence of oseltamivir-resistant influenza H1N1 strains (Leibbrandt et al., 2010).

The clinical effectiveness of carrageenan has also been reported against other viral infections including the common cold and human papilloma virus (HPV) (Buck et al., 2006; Koenighofer et al., 2014; Rodríguez et al., 2014). The administration of carrageenan nasal spray has been shown to increase viral clearance and decrease symptoms in children and adults with virus-induced common colds (Koenighofer et al., 2014). In addition, gel formulations of the sulfated polysaccharide showed significant anti-viral activities against HPV 16, HPV 18 and HPV 45 (Rodríguez et al., 2014). It is possible that carrageenan elicits this anti-viral activity against HPV by preventing the binding of the virus to specific sites on the host cell surface heparin sulfate which fuses the viral envelope with the host cell membrane and assists viral entry (Buck et al., 2006; Shukla and Spear, 2001). The effect of carrageenan on HPV was found to be three times more potent than heparin, a cell-free heparin sulfate therapeutic agent currently available in the market (Buck et al., 2006).

1.3.1.3 Anti-tumorigenic

Fucoidan, a sulfated polysaccharide extracted from a wide range of brown algal species including *Turbinaria tricostata*, (Chale-Dzul et al., 2015) *Sargassum crassifolium* and *Padina australis* (Yuguchi et al., 2016), has been demonstrated to possess promising therapeutic effects. For example, fucoidan extracted from the sporophylls of *Undaria pinnatifida* have been demonstrated to downregulate the levels of vascular endothelial growth factor (VEGF), a transcription factor critical in the proliferation of tumor cells, as well as inhibit lymphatic metastasis and cell invasion in a mouse hepatocarcinoma cell line (Teng et al., 2015). The anti-VEFG effect of fucoidan has also been demonstrated to inhibit metastasis in Lewis lung carcinoma and B16 melanoma in mice (Huang et al., 2015; Koyanagi et al., 2003).

Ascophyllan derived from *Ascophyllum nodosum* has also been reported to have potent anti-cancer effects on B16 melanoma cells (Abu et al., 2015). The mRNA expression and secretion of MMP-9 (matrix metalloprotease-9), which is well known to be abundant in tumor cells

(Simon et al., 1998), was suppressed in ascophyllan-treated melanoma cells. Ascophyllan also inhibited the migration of B16 melanoma cells, together with a decrease in the adhesion of tumor cells to type I collagen. This inhibitory effect was further demonstrated to have been elicited via a decrease in the expression of N-cadherin and an increase in E-cadherin production (Abu et al., 2015). Changes in levels of N-cadherin and E-cadherin are often associated with epithelial-mesenchymal transition (EMT), a dynamic process which is critical to tumor metastasis (Peinado et al., 2007). In addition, oral administration of the algal-derived polysaccharide has also been reported to exhibit antitumor effects in sarcoma-180 tumor mouse model, primarily through activating the host immune system (Jiang et al., 2014).

1.3.2 Food and agricultural applications

1.3.2.1 Functional food applications

Macroalgal species represent a major reservoir of high value compounds with potential application as functional food, amongst many others (Plaza et al., 2008). The variability of marine habitats with respect to environmental factors such as temperature, salinity, light absorption and pressure, ensures that seaweeds need to produce a wide range of bioactive compounds to protect against photodynamic damage, amongst many others. Research on the curation and characterization of these biologically active secondary metabolites from algae has been very well studied (Lordan et al., 2011) and considering the broad taxonomic range of seaweed species, it is clear that seaweeds and their bioactive compounds possess a huge potential in the food and agricultural sector.

Nowadays, there is an increased focus on the consumption of food with the additional benefits of disease prevention and health promotion, which go beyond satisfying our basic nutritional requirements. This category of food is known as functional food (Freitas et al., 2012). The need to manage or remedy several health problems including hypertension, diabetes, obesity, dyslipidemia and cardiovascular diseases which have been linked to dietary habits (Anand et

al., 2015; Mendonça et al., 2017; Schwingshackl et al., 2017; Song et al., 2016) have contributed to a greater demand for functional food.

Edible seaweeds are desirable as functional foods due to their high nutritional value and the presence of a number of high-valued compounds with specific health benefits (Lorenzo et al., 2017; Rupérez, 2002). While red and green algae are well known for their rich carbohydrate content, brown algae are abundant in soluble fiber and iodine, with levels of iodine ranging from 500 ppm in *Fucus* to 8000 ppm in *Laminaria* species (Gupta and Abu-Ghannam, 2011). The high mineral content of seaweeds also positions these marine species as a potential alternative to salt in processed meat products (Cofrades et al., 2011). The consumption of meat products has been reported to be associated with a higher risk of susceptibility to a number of health conditions such as coronary heart disease and stroke (Wolk, 2017). This has led to intense research on developing healthier meat and meat products which contain biologically active compounds and which can offer specific health promoting benefits, as well as enhancing the quality of the meat. For example, laminarin and fucoidan seaweed extracts have been reported to increase the antioxidant activity of cooked pork meat (Moroney et al., 2015), while the addition of three edible seaweeds to low-salt meat emulsion systems has also been shown to enhance their mineral content, amino acid profile and fatty acid composition (López-López et al., 2009). In other studies, macroalgal soluble and insoluble dietary fiber have been reported to improve the cost efficiency, texture and emulsion stability of meat products formulations (Fernández-Martín et al., 2009; Jiménez-Colmenero et al., 2010). Significant reduction in lipid oxidation in steak obtained from pigs which were fed with *Laminaria digitata* extracts containing laminarin and fucoidan in spray-dried (500 mg/kg feed) and wet forms (420 mg/kg) feed for 21 days has been demonstrated (Moroney et al., 2012), together with reports of a decrease in surface redness in cooked pork patties being elicited following exposure to spray-dried laminarin and fucoidan extracts (Moroney et al., 2013).These enriched meat products

were also in fact more preferable to the consumer, following a sensory analysis (Moroney et al., 2013).

Metagenomic and transgenic approaches are being employed to exploit the microbial diversity of seaweed-associated microbiome to enable the identification, biosynthesis and improved expression of marine-derived compounds which can offer potential health benefits. (Amiri-Jami and Griffiths, 2010) report the recombinant heterologous expression of fatty acids in *E. coli* cells as an alternative method for PUFA (poly unsaturated fatty acid) production by fermentation. Transgenic transformation of photoautotrophic algae into heterotrophic bioreactors for cost efficient overproduction of functional metabolites has also been proposed (Hallmann, 2007; Rasmussen and Morrissey, 2007). However, the future of these genetic modifications remain somewhat restricted due to both regulatory concerns and by public acceptance issues.

While the benefits of seaweeds as functional foods for improved health are undoubted, the acceptance and penetrance of these enriched food in the market are currently quite limited, primarily due to cultural and ethnic perceptions (Freitas et al., 2012). Edible seaweed represents a major dietary component in Asia regions including Korea, China and Japan. In Japan for example, supplementing pasta with the edible seaweed *Undaria pinnatifida*, which contains fucosterol and fucoxanthin responsible for improved fatty acid and amino acid profiles together with antioxidant activities, received good sensorial acceptance (Prabhasankar et al., 2009). Several other functional foods including noodles and potato chips with cholesterol-lowering functions are also available (Borderías et al., 2005). However, consumer acceptance in most European countries is limited by both culture and a perceived pessimism as to the real benefits of these new functional food. In addition, to the need to validate several claims and a small niche market insufficient for economic investments, regulatory regimes also create a bottleneck to the commercialization of functional food (Freitas et al., 2012; Holdt and Kraan, 2011).

1.3.2.2 Livestock farming applications

Seaweeds are being considered as an efficient alternative source of protein for animal feed production, as they eliminate the need for fertilizer and land usage required for the cultivation of land plants. The high protein content of seaweed species, together with their low lipid levels make them desirable to the livestock industry (Makkar et al., 2016). While ruminant livestock provide food for human consumption, livestock farming poses serious environmental challenges, with ruminants accounting for up to 14.5 % global methane emission (Gerber et al., 2013). Environmental sustainability concerns for livestock farming have thus led to increased research on strategies to decrease methane emission and improve feed conversion in ruminants, with the effects of phlorotannins, which are phenolic compounds exclusive to brown seaweeds, on ruminal fermentation being investigated. Tannins bind effectively to proteins and digestive enzymes in ruminants, thereby decreasing the ruminal fermentation of proteins, as well as carbohydrates (Vissers et al., 2018). The addition of phlorotannin extracted from *L. digitata* to grass silage at 40 g per kilogram, resulted in a 40% decrease in ruminal fermentation in cows and demonstrated the effectiveness of these macroalgal extracts in livestock farming. Phlorotannin has also been shown to effect an over 20% reduction in ammonia release and consequently increase the amount of protein available in the abomasum for digestion (Vissers et al., 2018).

Furthermore, seaweeds and seaweed extracts are being applied as alternatives to chemical additives to improve rumen function in animals. Several authors have reported the reduction effect of sun-dried *Ascophyllum nodosum* on faecal shedding of the pathogenic *E. coli* O157:H7 strain in cattle when supplemented with the animal diet (Bach et al., 2008; Barham et al., 2001; Braden et al., 2004). This finding has immense positive implications, as the asymptomatic abundance of pathogenic *E. coli* O157:H7 in cattle poses serious health concerns

and the risk of cross contamination of cattle products for human consumption (Bach et al., 2008).

1.3.2.3 Biostimulant market applications

Seaweed extracts are also gaining popularity in the biostimulant market which is valued at approximately US $2.5 billion, with *Ascophyllum nodosum* being the most commonly used seaweed species; as they elicit a range of effects on plants including improved chlorophyll synthesis, stress tolerance and root development (Goñi et al., 2016). Treatment of the model plant *Arabidopsis thaliana* with *A. nodosum* extracts has been shown to increase the number of rosette leaves in the plant and also induce early flowering (Goñi et al., 2016). Transcriptome analysis also revealed that the effects of *A. nodosum* extracts on the model plant were possibly elicited through the dysregulation of over 150 genes, some of which are involved in stress tolerance, vascular development, embryogenesis and nutrient transport. Furthermore, extracts from *A. nodosum* have been shown to enhance freezing tolerance and osmotic tolerance in grapevines (Mancuso et al., 2006; Wilson, 2001), as well as delaying wilting, increasing leaf water content and water use efficiency in vegetables (Battacharyya et al., 2015).

1.3.3 Biofuel and industrial applications

In recent times, there has been an increased demand for alternative renewable energy resources due to growing environmental concerns regarding the use of fossil fuels, as well as increases in crude oil prices (Daroch et al., 2013; Wei et al., 2013). Several studies have investigated the utilization of grain crops and lignocellulosic biomass as feedstock for the production of biofuel and have reported good yields (Liska et al., 2014; Kumar et al., 2009; Wortmann et al., 2010). However, these first and second generation biomass strategies compete with agricultural resources for human food consumption. On the other hand, marine macroalgae, which have a higher growth rate when compared to current land-based biomass (Munoz et al., 2004), thus possess a competitive advantage and are currently being studied as a third generation biomass

source for biofuel production, with the additional benefit of eliminating the need for fertilizer and arable land for cultivation (Daroch et al., 2013).

While seaweeds are rich in carbohydrates, low lipid levels may restrict the extraction of oils, which can be further processed into useful hydrocarbons, from these marine species (Ghadiryanfar et al., 2016). Nonetheless, macroalgae remain relevant to the biogas industry via anaerobic digestion, with numerous studies reporting very good levels of methane production from different seaweeds including *Ulva lactuca, Gracilaria vermiculophylla* and *Saccharina latissimi, with levels of* 271 L, 481 ± 9 L and 268 L CH_4/kg VS^{-1} respectively. The minimal presence or lack of lignin in algal biomass also offers an added advantage over land plants as they are more easily converted into simple sugars and also eliminates the need for expensive pre-treatment which are required to produce free cellulose for the subsequent bioconversion into biofuels such as ethanol (Daroch et al., 2013). Good yields of ethanol production from *Laminaria hyperborea, Sargassum fulvellum* and *Alaria crassifolia* feedstock have also previously been demonstrated (Daroch et al., 2013). The US Department of Energy estimates that up to 19,000 liters/ha/year can be generated from macroalgae, which corresponds to five times more ethanol that can be produced from corn (Wargacki et al., 2012). In addition, seaweeds are also rich in sugar alcohols such as mannitol which can be fermented by thermophilic bacteria, for example *Clostridia*, and can result in to up to an 88% theoretical yield of ethanol (Chades et al., 2018).

Significant variations have been demonstrated in the production of biomethane from seaweeds due to seasonal or morphological variations, as well as due to the algal species (Tabassum et al., 2016; Tabassum et al., 2018). These variations arise mainly due to differences in the biochemical composition of the macroalgae at different times of the year or in the different segments of the algal thallus. Lowest levels of ash in *Laminaria digitata* for example have been recorded in August, corresponding to the highest methane yield (327 L CH_4 kg VS^{-1}) being

observed (Tabassum et al., 2016). Similarly, the stipe region in *Saccharina latissima* which contained the highest levels of ash has been shown to result in lowest biomethane yields than the levels observed in the frond (Tabassum et al., 2018).

1.4 Brown algal cell walls

Brown algal cell walls share certain common features with land plants but also display unique properties. Unlike plant cell walls which comprise mainly of cellulose fibres, reaching up to 30% dry weight; the algal cell wall skeleton is generally made up of only 1% to 8% cellulose microfibrils (Deniaud-Bouët et al., 2014). The cell wall architecture in brown algae is organized into two main components including the skeleton and the matrix. Cellulose synthase complexes responsible for the synthesis of cellulose adopt a linear arrangement, rather than rosettes which are present in land plants. Hence they produce a flat bed of cellulose fibers up to 2.6 nm thick and 2.6-3.0 nm wide which forms the algal cell wall skeleton (Michel et al., 2010). The matrix component of brown algal cell walls is rich in anionic alginates and fucose-containing sulfated polysaccharides (FCSPs), together with phlorotannins which are synthesized in the Golgi apparatus and secreted onto the expanding cell wall (Michel et al., 2010). Sulfated polysaccharides which consist of α-l-fucosyl residues are referred to as sulfated fucans or fucose-containing sulfated polysaccharides (FCSPs) and their structure can vary from regularly homopolymers (homofucans) which primarily consist of a fucopyranose backbone as seen in *Saccharina latissima* and *Fucus serratus*, to highly branched heteropolymers (heterofucans) which contain high proportions of substituent groups such as mannose, rhamnose, xylose or galactose, as seen in *Sargassum fusiforme and* other *Sargassum spp* (Deniaud-Bouët et al., 2017). Studies reveal that sulphate residues on FCSPs modulate salinity levels (Torode et al., 2015) and contribute significantly to resistance to desiccation in algae, as a mode of adaptation to the intertidal environment where they are abundant (Mabeau and Kloareg, 1987).

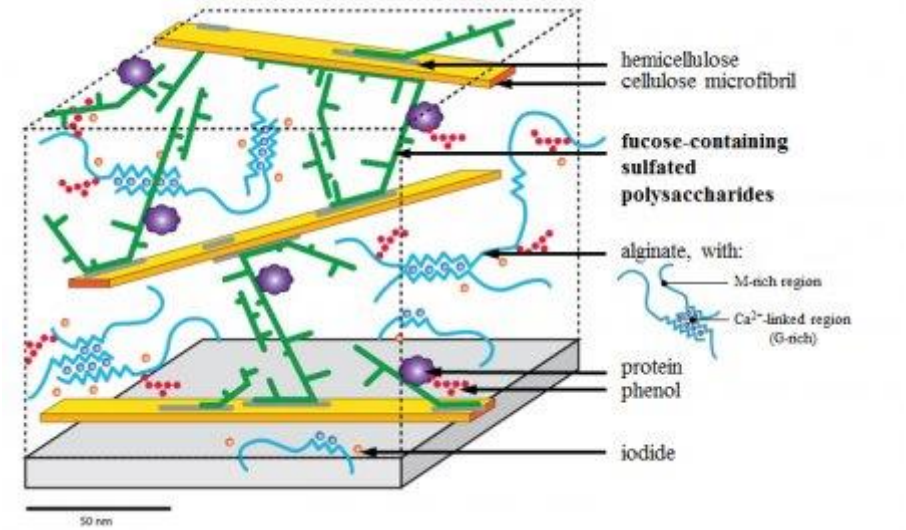

Figure 1.3: Brown algal cell wall model from the order Fucales. Adapted from (Deniaud-Bouët et al., 2014)

Recent experimental work on Fucales marked a milestone in the study of the architecture of brown algal cell walls and revealed significant insights (Figure 1.3) (Deniaud-Bouët et al., 2017). Sulfated fucans were shown to interlock cellulose microfibrils while the alginate components linked to phenolic compounds were also observed. This cell wall structure possibly contributes to the maintenance of the cell wall rigidity and to osmotic stress tolerance (Deniaud-Bouët et al., 2014; Torode et al., 2015). The work of Deniaud-Bouët and co-authors (Deniaud-Bouët et al., 2014) also provides additional information on glycoproteins, proteins, iodide and calcium ions which are present within the algal cell wall. In addition, recent extensive analysis of a number of brown seaweeds conducted by Salmeán and co-workers (Salmeán et al., 2017) have identified the presence of mixed linkage glucans (MLGs), which are a mixture of (1 → 3)-β- and (1 → 4)-β-linked glucose residues, in brown algal cell walls. While MLGs are well known to be found in green algae (Eder et al., 2008) and fungi (Burton and Fincher, 2009), the authors propose that the distinct identification of these polysaccharides in brown algae may have been masked by their linkage to alginates and are likely to provide strengthening benefits to the algal cell wall. In another study (Hervé et al., 2016), the presence

of a novel arabinogalactan protein (AGP) family in brown algal cell walls has been identified. These are densely glycosylated proteins that are rich in hydroxyproline residues and are located at the cell surface of plants. The structure of AGPs largely comprises of β-1,3-galactan chains, with the attachment of arabinose, rhamnose, glucuronic acid and other sugar residues to the end of β-1,6-galactan side chains (Hervé et al., 2016; Tan et al., 2012). Arabinogalactan proteins are suggested to play key functional roles relating to developmental process including embryogenesis and cell elongation in *Fucus serratus* (Hervé et al., 2016).

1.4.1 Prospects for exploiting algal cell walls

The complex nature of algal cell walls poses huge limitations with respect to the isolation and extraction of useful biologically active compounds, which are relevant to a wide variety of industries (some of these have previously been discussed), from macroalgae (Kadam et al., 2013; Synytsya et al., 2015). These algal derived bioactive compounds are quite sensitive to extraction technologies which involve high temperature, pressure and the use of organic solvents (Puri et al., 2012). In addition to this bio-sensitivity, the time-consuming and labour-intensive nature of traditional methods of extraction has led to a number of research studies which have investigated the possibilities of new alternative and more efficient procedures. In the past few decades, a range of such novel technologies have been developed, including microwave-assisted extraction, pressurized liquid extraction, supercritical fluid extraction, ultrasound-assisted extraction and enzyme-assisted extraction, each with unique benefits (Kadam et al., 2013). In particular, enzyme-assisted extraction (EAE), which involves the use of algal cell wall degrading enzymes to efficiently breakdown the cell wall barrier and contribute to the release of free and available phenolic compounds ready for extraction, offers an environmentally friendly and nontoxic alternative to conventional methods. Enzymes such as agarase, neutrase, alcalse, cellulase and viscozyme are commonly used in this procedure and several authors have reported the success of EAE technologies to obtain extracts from several

seaweeds. For example, bioactive compounds rich in rhamnose and uronic acids which displayed antioxidant and antiviral activities have been extracted from *Ulva armoricana* using enzymatic preparations (Hardouin et al., 2016).The enzymatic treatment of the green alga *Codium tomentosum* resulted in over 60% extraction yield of sugars (Rodrigues et al., 2015) while, antioxidant and antihypertensive protein extracts have recently been obtained from *Macrocystis pyrifera* using cellulases in EAE strategies (Vásquez et al., 2019). Enzyme-assisted extraction alleviates the water solubility barriers for desired biologically active compounds, as well as preserves the integrity of the extracted compounds to a great extent (Kadam et al., 2013). However, maximum extraction yields using EAE strategies largely depend on protocols which maintain optimum conditions such as pH, temperature, treatment times and the use of substrate-specific enzymes.

1.5 Marine macroalgae and the blue bioeconomy

As discussed in previous sections above, macroalgal species are rich in a wide range of biotechnologically relevant compounds which have a high commercial value, and are therefore significant contributors to the blue bioeconomy. However, in practice, the full exploitation of these marine species and the commercialization of some of their high value products has still yet to be fully achieved. In a recent report, the Blue Bioeconomy Forum (BBF) (Ligtvoet et al., 2019), consisting of marine researchers, entrepreneurs, government officials as well as other stakeholders, surveyed the blue bioeconomy landscape and identified several challenges which have hindered the advancement of marine species, including algae, into the marketplace. One of the main limitations identified is that while algal researchers may have discovered or developed potentially high-impact and innovative products in the laboratory, a limited understanding exists of the relevant investment options and funding opportunities as well as entrepreneurial skills required to commercialize these seaweed-derived products. The scarcity

of skills for technology transfer, product launch and the running of a successful business creates a bottleneck in the development of the blue bioeconomy, particularly with respect to marine algae. On the other hand, there is also a gap in the number of dedicated investment funds available to support blue bio projects and “start-ups”. In addition, up to 35% of participants in the recent BBF survey admit to facing difficulties in operation logistics such as handling, packaging, storage and transportation of algae and other marine biomass which have an impact on the overall deliverability of the sector from a biotechnological standpoint (Ligtvoet et al., 2019). Some of these challenges can however, be overcome by circumventing the barriers associated with the nature of marine biomass. For example, by employing innovative, faster and efficient technologies for drying seaweed, thereby affecting the time and resources required for storage and packaging. Furthermore, the tedious nature of licencing and permit applications has been found in some cases, to delay the advancement of seaweed and seaweed-derived products in the global commercial market. Therefore, the synchronisation of regulatory and legislative requirements, in addition to creating one-stop knowledge centres where free regulatory advice can be accessed, is recommended. Overall, it is important to note that the current limitations of the blue bioeconomy sector are interconnected and thus a holistic problem solving approach is required to further advancement (Ligtvoet et al., 2019).

1.6 Conclusion

In summary, marine macroalgae represent a promising resource for the isolation of novel biochemical compounds with a wide range of biotechnological applications. Of special interest in this study is the variations which occur within the complex and diverse algal associated bacterial communities. These microbes are likely to produce molecules, especially enzymes, with different biochemical properties from species found in terrestrial environments and are yet to be extensively explored.

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Chapter 2: Microbial population changes in decaying *Ascophyllum nodosum* result in macroalgal-polysaccharide-degrading bacteria with potential applicability in enzyme-assisted extraction technologies

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2.1 Abstract

Seaweeds are of significant interest in the food, pharmaceutical, and agricultural industries as they contain several commercially relevant bioactive compounds. Current extraction methods for macroalgal-derived metabolites are, however, problematic due to the complexity of the algal cell wall which hinders extraction efficiencies. The use of advanced extraction methods, such as enzyme-assisted extraction (EAE), which involve the application of commercial algal cell wall degrading enzymes to hydrolyze the cell wall carbohydrate network, are becoming more popular. *Ascophyllum nodosum* samples were collected from the Irish coast and incubated in artificial seawater for six weeks at three different temperatures (18 ˚C, 25 ˚C, and 30 ˚C) to induce decay. Microbial communities associated with the intact and decaying macroalga were examined using Illumina sequencing and culture-dependent approaches, including the novel ichip device. The bacterial populations associated with the seaweed were observed to change markedly upon decay. Over 800 bacterial isolates cultured from the macroalga were screened for the production of algal cell wall polysaccharidases and a range of species which displayed multiple hydrolytic enzyme activities were identified. Extracts from these enzyme-active bacterial isolates were then used in EAE of phenolics from *Fucus vesiculosus* and were shown to be more efficient than commercial enzyme preparations in their extraction efficiencies.

2.2 Introduction

Ascophyllum nodosum (L.) Le Jolis is a fucoid which is dominant along the intertidal rocky shores of the North Atlantic (Olsen et al., 2010). This brown macroalga is of great economic value as an important source of diverse bioactive compounds, many with valuable pharmaceutical, biomedical, and biotechnological potential (de Jesus Raposo et al., 2016); which include ascophyllan, laminarin, alginates, and polyphenols (Jiang et al., 2010; Nakayasu et al., 2009; Suleria et al., 2015). Extracts from *A. nodosum* have for example been reported to possess potent anticoagulant activity (Adrien et al., 2017), antitumor activity (Abu et al., 2015), anti-inflammatory activity (Zhang et al., 2014) together with antiviral activity (Wang et al., 2012), as well as possessing the ability to improve rumen function in animals (Belanche et al., 2016). Seaweed extracts are also known to help alleviate the consequences of abiotic stress in crops, with extracts from *A. nodosum* being reported to act at the transcriptional level in the model plant *Arabidopsis thaliana* (Goñi et al., 2016) and to also alleviate drought stress in this plant species (Santaniello et al., 2017). Thus, seaweeds and their useful derivatives have become subject to extensive research in recent times.

Macroalgal surfaces are well known to provide a suitable substratum for the attachment of microbial colonizers, including fungi and bacteria, with bacterial densities reaching levels ranging from 10^2 to 10^7 cells cm^{-2} (Bengtsson et al., 2010). Organic substances secreted by the macroalga act as an important nutritional source for these microorganisms (Egan et al., 2013; Singh et al., 2013; Steinberg et al., 2002). Epiphytic bacterial communities are in fact believed to be essential for normal morphological development in the algal host, with these bacteria producing chemicals which help to protect the macroalga from potential harmful secondary colonization by pathogenic microorganisms (Goecke et al., 2010; Singh and Reddy, 2014). It is believed that some algal species may contain distinct associated bacterial communities, related to the composition of the algal surfaces and their exudates (Lachnit et al., 2013). Several

environmental and non-environmental factors have been shown to influence the composition and abundance of such epibacterial communities associated with seaweeds. In addition to seasonal and temporal variations, the physiological state of the macroalga has also been found to play a significant role in the structure of algal associated microbial communities. Recent studies on *Cladophora*, a filamentous green alga, revealed changes in the diversity and composition of its associated epibacterial communities during decay, to include species involved in nutrient recycling (Chun et al., 2017).

Marine bacteria are likely to produce cell-wall degrading enzymes as a mechanism to mobilize polymers for nutritional purposes when growing in a nutrient limited state, such as growth on decaying algae. It has been proposed that macroalgal-polysaccharide-degrading (MAPD) bacteria will increase in numbers on weakened or dead macroalgae, thus contributing to recycling of the algal biomass (Martin et al., 2015). Several algal polysaccharide-degrading bacteria, which were taxonomically assigned to the *Flavobacteria* and γ-*Proteobacteria* classes have recently been isolated from the microflora of *A. nodosum* (Martin et al., 2016). These bacteria displayed diverse hydrolytic activities and the subsequent functional screening of plurigenomic libraries from these bacteria resulted in the discovery of a range of novel hydrolytic enzymes (Martin et al., 2016). Thus, it is clear that the diverse and complex bacterial communities associated with *A. nodosum* represent a potential source of novel hydrolytic enzymes with biotechnological applications that could include enzyme-assisted extraction (EAE) strategies and improvements in the yields of algal components with cosmeceutical, functional food, nutraceutical, and biopharmaceutical applications (Hardouin et al., 2016; Kulshreshtha et al., 2015).

While seaweeds are rich in useful polysaccharides and other metabolites (Leal et al., 2013), extraction of such algal components can be problematic due to the complexity and rigidity of

the algal cell wall. Brown algal cell walls consist of complex sulfated and branched polysaccharide bound to proteins and ions that hinder extraction efficiencies of algal-derived metabolites (Deniaud-Bouët et al., 2014). Chemical and mechanical processes are currently used for the extraction of bioactive compounds or fractions from algae; however, problems exist if these compounds are sensitive to extraction techniques which involve heat or the use of solvents (Kadam et al., 2013). New improved extraction processes including microwave, ultrasound, supercritical fluid, pressurized liquid, and particularly the use of enzyme-assisted extraction (EAE) processes (Kadam et al., 2013), offer ecofriendly, faster, and more efficient alternatives to traditional method (Hardouin et al., 2016; Jeon et al., 2011; Joana Gil-Chávez et al., 2013). Commercial algal cell wall polysaccharidases, such as xylanase, alcalase, viscozyme, neutrase, and agarase, can be used to hydrolyze algal cell wall carbohydrates and eliminate solubility barriers for algal bioactive compounds (Kadam et al., 2013; Nadar et al., 2018). Enzyme-assisted extraction (EAE) has been successfully employed to produce extracts from the green seaweed *Ulva rigida* (formerly *Ulva armoricana*), which displayed antiviral and antioxidant properties (Hardouin et al., 2016). Carbohydrates and bioactive compounds have also been extracted from the brown algae *Ecklonia radiata* and *Sargassum muticum*, respectively, using carbohydrate hydrolases and proteases in EAE strategies (Charoensiddhi et al., 2016).

In this study, our approach was to monitor the overall composition and population dynamics of the microbial communities associated with *A. nodosum* as it decayed over a six-week period by incubating the alga in artificial seawater, using culture independent approaches, targeting the 16S rRNA gene. We also employed culture dependent approaches, including the ichip method (Nichols et al., 2010) to isolate bacteria from the seaweed during the decay process. While bacteria have previously been isolated from macroalgae using traditional cultivation methods (Boyd et al., 1999; Martin et al., 2015), this study presents the first use of the ichip to

cultivate bacteria from decaying seaweed. In this way, we hoped to expand the range of bacterial isolates to include previously uncultured microorganisms. Following isolation, bacterial strains were screened for their ability to produce a range of algal cell wall degrading polysaccharidases and were characterized by 16S rRNA DNA sequencing. A range of species from the genus *Bacillus*, together with a number of *Vibrio* species, were isolated, these displayed multiple hydrolytic enzyme activities including hydroxyethyl cellulase, lichenase, and pectinase activities. Extracts from these bacteria were then successfully employed in the EAE of phenolics from the seaweed *Fucus vesiculosus*.

2.3 Results

2.3.1. MiSeq Sequencing and Data Processing

The microbial communities of *A. nodosum* samples were analyzed based on decay period (week 0, week 2, week 4, and week 6) and incubating temperature (18 ˚C, 25 ˚C, and 30 ˚C) by Illumina MiSeq sequencing targeting the V3-V4 16S rRNA gene region. A combined total of 8,872,164 raw reads were obtained which, when quality filtered produced, 1,655,910 reads with an average length of 301 bp, and were analyzed using the QIIME version 1.9.1 (http://qiime.org/tutorials/illumina_overview_tutorial. html) workflow (Caporaso et al., 2010). The number of reads obtained after quality filtering and operational taxonomic units (OTUs) together with species richness and diversity indices of the microbial communities are shown in Table 2.1. With respect to the two diversity indices calculated; Shannon and Chao1, sample 4_30 (week 4; 30 ˚C) ranked the highest and sample 6_18 (week 6; 18 ˚C) ranked the lowest in bacterial diversity. In addition, it is noteworthy that up to 4% of the OTUs observed in the undecayed *Ascophyllum* sample (T_0; week 0) and 41% in the decaying samples were unclassified. These unassigned OTUs are likely to represent macroalgal associated bacterial populations that are as yet unknown or not present in the SILVA version 123 database (Quast et al., 2012) used for taxonomy assignment.

Table 2.1. Observed OTUs and species richness and diversity estimates of *Ascophyllum*-associated metagenomic communities obtained using MiSeq sequencing of the 16S rRNA gene from the intact seaweed (T_0) and each of decaying *Ascophyllum nodosum* samples collected at three phases of the decay period (week 2, week 4 and week 6) at 18 ˚C (2_18, 4_18, 6_18), 25 ˚C (2_25, 4_25, 6_25), and at 30 ˚C (2_30, 4_30, 6_30).

Decay Period	Sample	No. of Reads after Quality Filtering	No. of OTUs (at 97% Sequence Identity)	Chao1 Richness	Shannon Index
Week 0 intact seaweed	T_0	178,699	1467	2293.5	10.1
Week 2 early decay phase	2_18	123,551	854	2072.1	9.5
	2_25	350,135	1476	3327.1	10.2
	2_30	138,445	724	1822.7	9.2
Week 4 mid decay phase	4_18	148,904	749	1901.7	9.3
	4_25	151,285	1130	2061.6	9.8
	4_30	139,737	1633	3490.4	10.4
Week 6 late decay phase	6_18	120,679	443	1148	8.6
	6_25	138,659	1202	2884.5	10.0
	6_30	165,816	1250	3062.8	10.0

2.3.2. Metagenomic Communities Associated with Intact Ascophyllum nodosum

A total of 1467 OTUs were identified among sequence reads derived from the intact undegraded seaweed (T_0), with approximately 96% of the OTUs been classified into one of 19 different phyla, the major ones being *Proteobacteria* (24.2%), *Planctomycetes* (22.5%), *Actinobacteria* (15.2%), *Verrucomicrobia* (15.1%), *Cyanobacteria* (6.7%), *Bacteroidetes* (4.8%), and *Firmicutes* (3.8%) (Figure 2.1; T_0). The species richness and diversity of the microbial community associated with the macroalga in its intact state as represented by the

number of OTUs and number of bacterial phyla identified is also reflected by the Chao1 richness and Shannon diversity index calculated (Table 2.1).

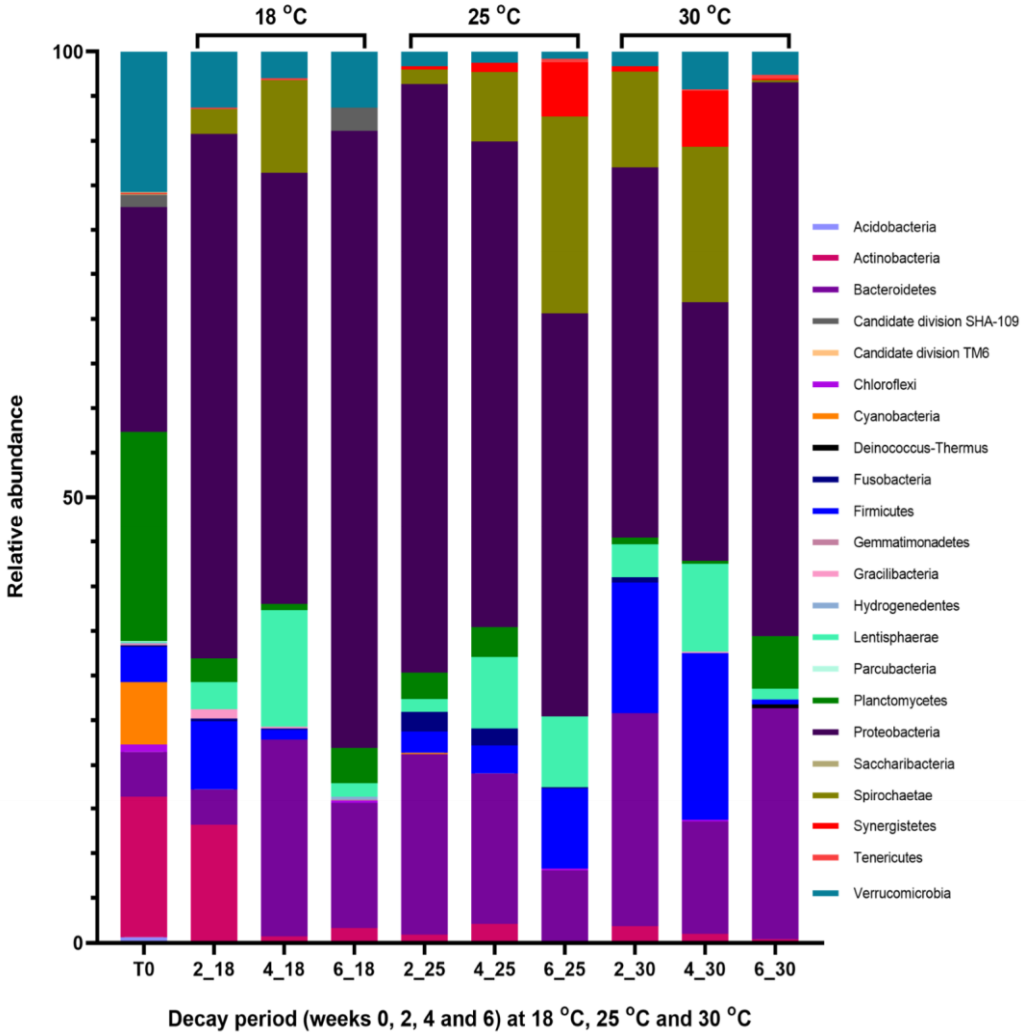

Figure 2.1. Relative abundances of bacterial phyla associated with intact (T_0) and decaying *Ascophyllum nodosum* at 2, 4, and 6 weeks of decay at 18 ˚C (2_18, 4_18, 6_18); 25 ˚C (2_25, 4_25, 6_25), and 30 ˚C (2_30, 4_30, 6_30) obtained from metagenomic 16S rRNA gene sequencing. The relative distribution of phyla in each group is represented as a percentage.

2.3.3. Metagenomic Communities Associated with Decaying Ascophyllum nodosum

2.3.3.1. Population Changes in the Seaweed Decaying at 18 ˚C

Within the first two weeks of the algal decay at 18 ˚C, the bacterial population associated with the intact seaweed, which was previously characterized by the prevalence of *Proteobacteria* (24.2%), *Planctomycetes* (22.5%) and *Verrucomicrobia* (15.1%), shifted towards a *Proteobacteria*-led population with a relative abundance of over 40% (Figure 2.1; 18 ˚C). This increase in the prevalence of *Proteobacteria* occurred with a corresponding decline in the relative abundances of some phyla including *Planctomycetes* (1.8%) and *Verrucomicrobia* (4.3%), amongst others. However, in the early decay period (at week 2), bacteria belonging to the phylum *Spirochaetae* which were not identified in the intact seaweed emerged, while other phyla such as *Firmicutes, Fusobacteria*, *Lentisphaerae*, and *Gracilibacteria* were found to be increasingly abundant, relative to their levels prior to the algal decay. The composition of the metagenomic communities associated with the decaying seaweed remained unchanged in the next phase of the algal decay process (at week 4), but changes in the relative abundances of some phyla occurred. At week 4, increase in the presence of bacterial groups classified as *Bacteroidetes, Spirochaetae*, and *Lentisphaerae* were more apparent while *Proteobacteria* levels declined. The bacterial population identified at the end of the decay process (week 6) was less diverse, with 443 OTUs identified and low diversity indices calculated (Table 2.1). Metagenomic results show that a number of bacterial phyla present in the preceding decay phases were not found at week 6. Some of these bacterial phyla which were not present include *Firmicutes, Fusobacteria, Spirochaetae* and *Gracilibacteria*.

2.3.3.2. Population Changes in the Seaweed Decaying at 25 ˚C

When compared to the bacterial population associated with fresh *Ascophyllum nodosum* samples, the macroalga allowed to decay at 25 ˚C experienced a sharp decrease in the relative abundances of bacteria belonging to the phyla *Planctomycetes, Verrucomicrobia, Firmicutes* and *Actinobacteria* amongst others in the early decay period. Metagenomic analysis of the 16S rRNA gene sequences revealed a steady shift in the composition and abundance of the

microbial communities associated with the decaying seaweed. For example, while the phylum *Spirochaetae* was not identified prior to decay (T_0), bacteria classified as this phylum were identified at week 2 (0.9%) and increased in relative abundance throughout the decay period from 3.7% at week 4 to approximately 11% at week 6. *Lentisphaerae*, which was rarely identified in the undecayed macroalga, was also observed to follow a similar trend, by increasing in relative abundance as *A. nodosum* samples decayed at 25 ˚C. Levels of this phylum increased from 0.8% at week 2 to 3.8% and 4% at the mid and late phases of the decay period, respectively. Other phyla such as *Fusobacteria* and *Bacteroidetes* were observed to decrease consistently in their prevalence from week 2 to week 6 of the decay process (Figure 2.1; 25 ˚C). In general, *Ascophyllum nodosum* allowed to decay at 25 ˚C was dominated by bacteria recruiting to the phylum *Proteobacteria*.

2.3.3.3. Population Changes in the Seaweed Decaying at 30 ˚C

At 30 ˚C, the decaying macroalga was diversely comprised of bacteria belonging to different phyla (Figure 2.1; 30 ˚C). The seaweed-associated metagenomic population changed markedly upon decay, relative to the microbial communities found in the intact seaweed (described in Section 2.3.2) and some of the most notable changes observed occurred in the early decay phase. At week 2, the relative abundance of bacteria belonging to the phylum *Actinobacteria* was observed to have decreased from approximately 15% (found in week 0) to about 1%. This decrease in the relative abundance of *Actinobacteria* was observed to be concurrent with a decline in the prevalence of bacteria belonging to the phyla *Planctomycetes* (0.4%) and *Verrucomicrobia* (0.9%) previously present at approximately 22% and 15%, respectively, in the intact seaweed. On the other hand, in the early decay phase, other phyla such as *Bacteroidetes, Lentisphaerae,* and *Firmicutes* were observed to increase to more than two-fold in their relative abundances in the decaying seaweed. The microbial population observed at the mid decay phase (week 4) did not differ greatly in its composition from the week 2 derived

microbial population. However, the prevalence of *Proteobacteria* declined (22.4% early phase; 17.1% mid phase) as bacteria identified as *Spirochaetae* (5.8% early phase; 10.3% mid phase) and *Synergistetes* (0.3% early phase; 3.7% mid phase) increased in prevalence. Differences in the bacterial phyla present in the decaying seaweed became even more evident at the end of the decay period (week 6) when *Proteobacteria* regained dominance (43.1%) and the levels of *Spirochaetae* (0.2%) and *Synergistetes* (0.1%) declined.

2.3.4. Cultivable Surface Microbiota Associated with Intact A. nodosum

The cultivable epibacterial population of intact and decaying *A. nodosum* samples were assessed using the maceration cultivation method, which involves cutting the seaweed samples into smaller fine pieces. A total of 90 bacteria were isolated and taxonomically identified following 16S rDNA sequence analysis (Table 2.2) from the intact *Ascophyllum nodosum* sample (T_0) and were found to consist of bacteria belonging to the phyla *Proteobacteria* (46%), *Bacteroidetes* (43%) and *Actinobacteria* (11%) (Figure 2.2a). Members of the phylum *Proteobacteria* were largely dominated by the class γ–*Proteobacteria* (95%), with α-*Proteobacteria* and β-*Proteobacteria* being rarely isolated. In the total isolated bacterial population from the intact seaweed (T_0), eleven different genera were identified, the most abundantly represented being *Winogradskyella* (41%), *Marinobacter* (37%), *Microbacterium* (6%), and *Micrococcus* (6%) (Figure S2.1a; Supplementary information).

Table 2.2**.** Number of bacterial isolates cultured from intact (week 0) and decaying *Ascopyllum nodosum* samples (week 2, week 4, and week 6), incubated at different temperatures, using the maceration isolation method and the ichip device.

Week 0	Week 2	Week 4	Week 6	ichip Isolation	Incubating Temperature
	76	63	52	59	18 ˚C
90	35	47	70	76	25 ˚C
	63	67	53	89	30 ˚C

2.3.5. Cultivable Surface Microbiota Found on Decaying A. nodosum

The cultivable surface-attached microbiota of the intact seaweed differed greatly from the bacterial populations found on *Ascophyllum* samples allowed to decay at 18 ˚C, 25 ˚C, and 30 ˚C for six weeks (Figure 2.2). At the phylum level, while *Proteobacteria* maintained an overall dominance with over 70% relative abundance in the bacterial population associated with both the intact and decaying seaweed, members of the phylum *Firmicutes* which were not identified in the intact seaweed were present during the algal decay. Bacteria belonging to the phylum *Bacteroidetes*, which were prevalent in the intact macroalga (43%), represented only 3% of the total cultivable surface microbiota population in the decaying seaweed.

Similar bacterial phyla were present in the bacterial communities associated with the decaying seaweed in the three incubation flasks (18 ˚C, 25 ˚C, and 30 ˚C). However, distinct differences in the composition of the associated microbial populations found at the different decay periods and temperature are more evident at the genus level (Figure S2.1; Supplementary information). In the early decay phase, the bacterial population isolated from the macroalga decaying at 18 ˚C consisted of *Proteobacteria* (88%), *Firmicutes* (5%), *Bacteroidetes* (4%), and *Actinobacteria* (3%) (Figure 2.2b) and was diversely comprised of members of the genera;

Paracoccus (71%), *Celeribacter* (7%), *Psychrobacter* (8%), *Bacillus* (5%), *Formosa* (4%), *Microbacterium* (3%), *Cobetia* (1%), and *Citricella* (1%) (Figure S2.1b; Supplementary information). Much lower diversity was observed in the bacterial communities isolated at week 2 from the 25 ˚C and 30 ˚C incubation flasks, with only members of the phyla *Proteobacteria* and *Firmicutes* being identified.

In the mid decay period (week 4), the genus *Celeribacter* dominated the bacterial population with a relative abundance of 95% and 100% in the 18 ˚C and 30 ˚C derived bacterial populations, respectively, while the 25 ˚C derived population at week 4 was comprised of more genera including *Paenisporosarcina* (51%), *Bacillus* (30%), *Celeribacter* (13%), *Paenibacillus* (2%) *Vibrio* (2%), and *Sporosarcina* (2%) (Figure S2.1c; Supplementary information). Members of the bacterial phyla *Proteobacteria* (54%), *Bacteroidetes* (27%), and *Firmicutes* (19%) were identified in the bacterial population cultured at the end of the decay process (week 6) from the 18 ˚C incubation flask. These phyla were also present at 72%, 7%, and 21% relative abundances, respectively, in the 30 ˚C bacterial population (Figure 2.2d). In week 6, eleven distinct genera were identified at 18 ˚C, the most abundantly represented being *Paracoccus*, *Algoriphagus*, *Celeribacter*, *Bacillus*, and *Primorskyibacter* and four genera including *Celeribacter*, *Bacillus, Pseudozobellia*, and *Paracoccus* being observed in the 30 ˚C microbial community. The bacterial community associated with the seaweed decaying at 25 ˚C in the late decay phase differed from the bacterial communities isolated from the 18 ˚C and 30 ˚C microbial populations in this phase, with *Proteobacteria* (96%) dominating the dataset and three genera; *Celeribacter, Bacillus* and *Roseobacter* being isolated at 25 ˚C. Phylogenetic trees representing the bacteria cultured from both intact and decaying *Ascophyllum nodosum* samples using the maceration method are shown in Figures S2.2–S2.5 (Supplementary information).

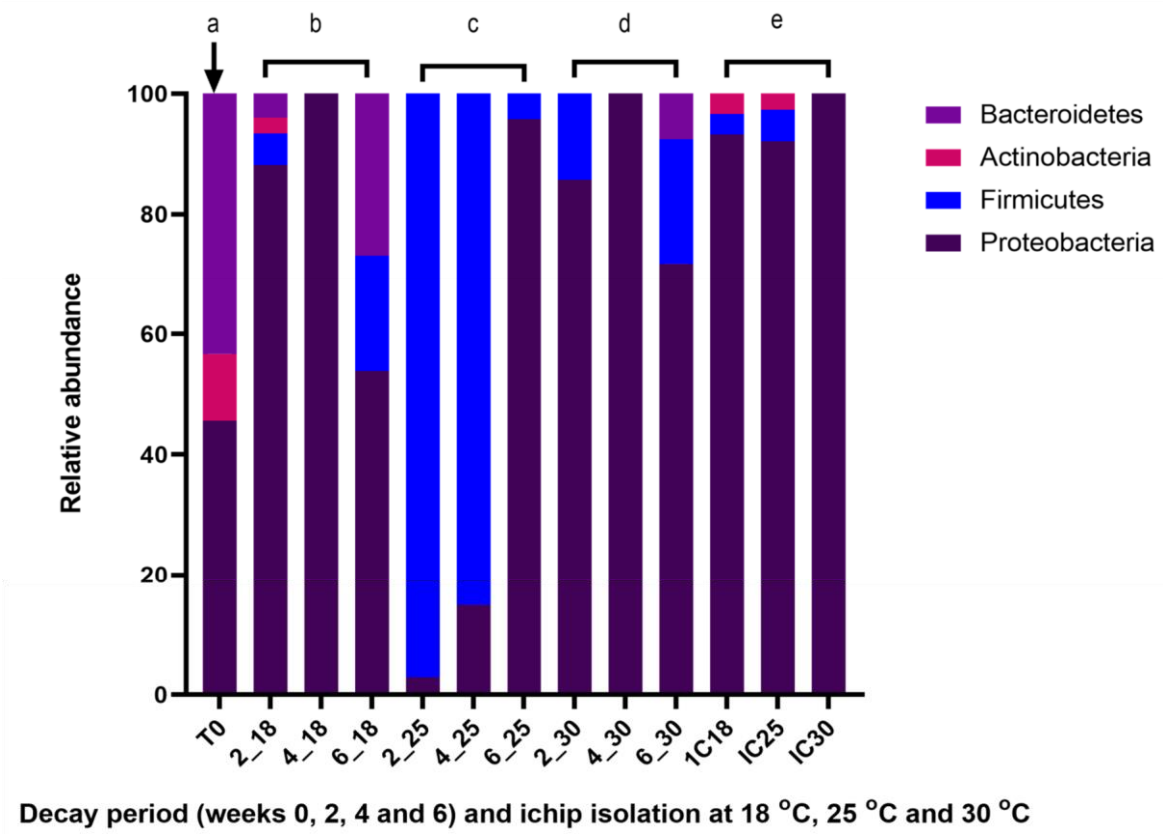

Figure 2.2**.** Relative abundances of bacterial phyla associated with the cultivable surface microbiota of (**a**) intact *Ascophyllum nodosum* and decaying *Ascophyllum nodosum* at 2, 4, and 6 weeks of decay at (**b**) 18 ˚C; 2_18, 4_18, 6_18, (**c**) 25 ˚C; 2_25, 4_25, 6_25, (**d**) 30 ˚C; 2_30, 4_30, 6_30 which were obtained by maceration culture isolation method and (**e**) obtained by ichip culture isolation method. 16S rRNA gene sequences were obtained from the bacterial isolates and taxonomic analyses were performed. The relative distribution of phyla in each group is represented as a percentage.

2.3.6. ichip Bacterial Isolation Method Applied to Decaying A. nodosum

In a bid to further analyze the cultivable bacteria present and potentially expand upon the range of bacterial isolates, the ichip device was also employed on the decaying seaweed samples. At week 4 of the decay period, the ichip device loaded with a cell–agar suspension prepared from the decaying seaweed was inoculated into each flask containing the alga which were decomposing at 18 ˚C, 25 ˚C, and 30 ˚C; with bacteria being recovered from the device

following a further 2 weeks of incubation. A total of 224 bacteria (59 isolates from 18 ˚C, 76 and 89 isolates from 25 ˚C and 30 ˚C, respectively; Table 2.2) were isolated and taxonomically identified using 16S rRNA gene sequences. Taxonomic analysis of the cultivable microbial communities revealed the presence of three representative phyla—*Proteobacteria*, *Actinobacteria*, and *Firmicutes*—across the three different temperature groups. *Proteobacteria* dominated at the phylum level in the three datasets, comprising 93%, 92%, and 100% in the 18 ˚C, 25 ˚C, and 30 ˚C bacterial culture populations, respectively (Figure 2.2e). Low relative abundances of *Actinobacteria* and *Firmicutes* were observed in the 18 ˚C and 25 ˚C populations, with both phyla not being identified in the 30 ˚C bacterial population. The majority of bacterial isolates cultured from the 18 ˚C and 25 ˚C samples, which belong to the phylum *Proteobacteria*; were further classified as α–*Proteobacteria*, with only 9–11% recruiting to γ-*Proteobacteria*. In contrast, γ-*Proteobacteria* were found to dominate the microbial community isolated from the seaweed incubated at 30 ˚C, with a relative abundance of 98%. Thirteen distinct genera, including *Celeribacter*, *Paracoccus*, *Vibrio* and *Marinobacterium* were present in the total bacterial population isolated using the ichip device (Figure S2.1e; Supplementary information). The genus *Celeribacter* was present across all temperatures, at very high relative abundances in the 18 ˚C and 25 ˚C samples (64% and 83%, respectively) but at a much lower relative abundance at 30 ˚C (2%). In contrast, *Enterobacter*, which dominated at 30 ˚C, was not found to be present in either of the 18 ˚C or 25 ˚C derived microbial populations. The phylogenetic tree representing the bacteria cultured from decaying *Ascophyllum nodosum* samples using the ichip in situ cultivation method is shown in Figure S2.6 (Supplementary information).

2.3.7. Enzymatic Activities of A. nodosum *Cultivable Surface Microbiota*

2.3.7.1. Intact *Ascophyllum nodosum* Isolated Using the Maceration Method

Over 800 bacterial isolates cultured from intact (T_0) and decaying *Ascophyllum nodosum* samples using both the maceration and ichip isolation methods (Table 2.2) were screened for enzyme activity in plate assays containing hydroxyethyl cellulose, pectin, and lichenin as substrates. The cultivable surface microbiota community associated with the seaweed in its intact state was found not to produce any of the algal cell wall degrading enzymes examined under the conditions employed in this study, with none of the bacterial isolates testing positive on any of the plate assays used.

2.3.7.2. Decaying *Ascophyllum nodosum* Isolated Using the Maceration Method

The microbial population associated with the decaying seaweed isolated using the maceration method consisted of a total of 51 isolates (approximately 7%) with hydrolytic activity against at least one of the tested substrates (Table S2.1, Supplementary information). Of these enzyme active bacterial isolates, 65% belonged to the microbial community cultured from the decaying seaweed at week 2, another 10% belonged to the week 4 bacterial population, while 25% were cultured from week 6 and the majority of these MAPD bacteria were found to degrade lichenin (Table S2.1; Supplementary information). Bacteria belonging to the genus *Bacillus* (10%) represented one of the less abundant genera in the total microbial community associated with the decaying seaweed. However, among the bacteria cultured from the decaying seaweed using the maceration method, these *Bacillus* species were found to be the only producers of the algal cell wall polysaccharidases tested for in this study.

2.3.7.3. Decaying *Ascophyllum nodosum* Isolated Using the ichip Method

Approximately 5% of the ichip-derived microbial communities screened were identified as being positive for one or more of HE-cellulose, lichenin, and pectin degrading activities. None of the bacterial isolates from 30 ˚C displayed MAPD activity under the conditions tested in this study while less than 3% of the 25 ˚C derived population tested positive and 15% from the 18 ˚C bacterial population were enzyme active. All the enzyme active bacterial isolates cultured

from 18 °C were identified as belonging to the *Vibrio* genus. These isolates were found to produce pectin degrading enzymes (Table S2.1; Supplementary information).

2.3.8. Enzyme-Assisted Extraction (EAE) of Total Phenolics from F. vesiculosus

We then compared the ability of an enzymatic bacterial supernatant (EBS) generated from the three isolates IC18_D7 (DSM 107285), IC18_D5 and ANT_0_A6 (DSM 107318) with ≥98% 16S rRNA gene sequence similarity to *Vibrio anguillarum* X0906, *Vibrio oceanisediminis* S37, and *Winogradskyella sp.* MGE_SAT_697, respectively, which we had selected as the best enzyme producers from our group of enzyme-active strains to perform enzyme-assisted extraction of phenolics from *Fucus vesiculosus*, and to compare their performance to commercially available enzyme preparations. Bacterial isolates IC18_D7 and IC18_D5 were shown to produce pectin degrading enzymes. ANT_0_A6 had previously been shown to produce good levels of amylase activity (data not shown). Results obtained from the EAE of the total phenolic compounds from *F. vesiculosus*, performed with or without commercial enzymes conducted at 50 °C, and with or without the enzymatic bacterial supernatants (EBS) conducted at 28 °C, are shown in Figure 2.3. The total phenolic content (TPC) of *F. vesiculosus* obtained by exhaustive solid-liquid extraction had previously been reported as 68.6 ± 8.3 mg PE.g^{-1} DWB (Kenny et al., 2015). This content was thus considered as a reference value for TPC, corresponding to a yield of extraction of 100%. Although the highest TPC values were obtained using commercial enzymes, compared to the control (50 °C), the increase was only significant when xylanase was used on the larger biomass particles i.e., 0.5 < Ps < 2.5 mm (p = 0.021). This TPC value of 35.6 ± 2.0 mg PE.g^{-1} DWB, obtained using xylanase, was equivalent to an extraction yield of 52%. Using the enzymatic bacterial supernatants (EBS), the TPC values increased significantly for both particle sizes (p < 0.01), compared to the control (28 °C), reaching up to 44.8 ± 1.8 mg PE.g^{-1} DWB (Ps < 0.5 mm) and 40.3 ± 1.7 mg PE.g^{-1} DWB (0.5 < Ps < 2.5 mm), respectively. These TPC values correspond to extraction yields of 65% and

59%, respectively. The extraction yields were therefore increased by 10% using xylanase, while they increased by 11–13% using EBS, compared to their respective controls. Moreover, an increase in extraction temperature (control 28 ˚C vs control 50 ˚C) appeared to have an overall negative effect on the extraction yield for phenolics. These results indicate that cell-wall degrading enzyme preparations produced by the three bacterial isolates from *A. nodosum*, applied at 28 ˚C were more efficient than the commercial protease, cellulase and xylanase preparations in the extraction of total phenolics from *F. vesiculosus*.

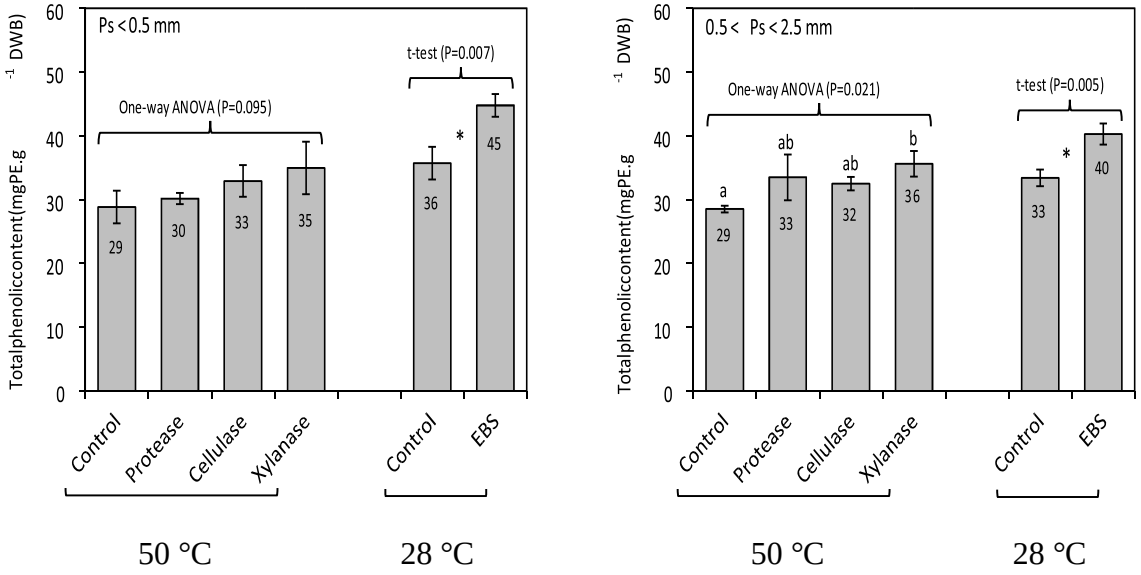

Figure 2.3**.** Enzymatic-assisted extraction of total phenolics from *Fucus vesiculosus* with commercial enzymes conducted at 50 ˚C, and with enzymatic bacterial supernatants (EBS) conducted at 28 ˚C. Control experiments without the addition of either commercial enzymes or EBS were conducted at 50 ˚C and 28 ˚C, respectively, under the same conditions. This experiment was undertaken using two different particle sizes (Ps) of ground biomass i.e., Ps < 0.5 mm, and 0.5 < Ps < 2.5 mm. Total phenolic content (TPC) is expressed as milligram of phloroglucinol equivalents (PE) per gram of dry weight biomass (mg PE.g^{-1} DWB). A one-way ANOVA was performed to assess significant differences (p < 0.05) between commercial enzymes and control (50 ˚C), results are arranged in

increasing order: a < b; while a t-test was performed to determine significance differences ($p < 0.05$) between EBS and control (28 ˚C), asterisk (*) indicates a difference between both treatments.

2.4. Discussion

Macroalgal bioactive compounds are used in products to stimulate animal health or as functional food ingredients (Holdt and Kraan, 2011; Joana Gil-Chávez et al., 2013; Pulz and Gross, 2004). Phlorotannins exclusive to brown algae in high amounts (15% DW) have been shown to possess antidiabetic (Lee and Jeon, 2013), antioxidant (Shibata et al., 2007), and antiproliferative (Nwosu et al., 2011) effects. In addition, seaweed extracts are commonly used as biostimulants in agriculture (Craigie, 2011) and have been proposed as a viable alternative protein crop for use in diets for monogastric livestock (Angell et al., 2016).

Seaweeds are well known to be associated with a diverse range of bacteria which colonize their nutrient-rich surfaces (Bengtsson et al., 2010; Martin et al., 2015; Martin et al., 2016). These bacteria are known to be a very good source of specific polysaccharidases, including pectinases, alginate lyases, carrageenanases, fucoidanases, and laminarinases (Martin et al., 2014) with several biotechnological applications. Some of these algal cell-wall degrading enzymes are produced to help mobilize polymers for nutritional purposes, for example, when growing in a nutrient limited state such as algal decay, and contribute to algal biomass recycling (Chun et al., 2017; Martin et al., 2015). Thus, we reasoned that if *A. nodosum* was allowed to decay under controlled conditions at different temperatures, it should result not only in changes in the overall composition and dynamics of the bacterial communities present, but also in the isolation of bacteria that produce algal cell wall polysaccharidases, given the nutrient limited state to which they had been exposed, that might have potential application in EAE strategies.

The structure of the surface-attached bacterial population associated with intact and decaying *A. nodosum* incubated at 18 ˚C, 25 ˚C, and 30 ˚C was investigated in this study using both

culture independent and culture dependent (traditional maceration and the in-situ cultivation based ichip device) approaches. The use of a next-generation sequencing approach (Illumina MiSeq) supplemented the 16rRNA gene-based approach employed on the cultured bacterial isolates. Given that the NGS approach circumvents the difficulties associated with the cultivation of bacteria from environmental samples and allows the identification of both cultivable and non-cultivable bacterial populations, it is not surprising that some phyla observed in the metagenomic communities of the macroalga were not identified in the total cultivable bacterial population. In particular, considering the isolation agar (SYP-SW) and the culture condition (72 h at 28 ˚C) employed in this study, it is highly unlikely that most phyla, including *Planctomycetes*, *Spirochaetae*, and *Verrucomicrobia*, which were found in the NGS dataset, would be recovered. These bacterial phyla would require a more targeted isolation strategy to be identified using various plate-based cultivation methods (Dubinina et al., 2015; Jeske et al., 2015; Lage and Bondoso, 2012; Tanaka et al., 2017).

The ichip device, which has previously been reported to increase the microbial diversity of cultured bacterial isolates (Berdy et al., 2017; Kealey et al., 2017; Ling et al., 2015), was applied to potentially expand the range of bacterial isolates identified to include previously uncultured species. While the composition of the microbial communities derived from the ichip device did not differ greatly from the bacterial populations identified using the traditional approach (Figure 2.2), we recovered four potentially novel strains (IC25_B4, IC25_B12, IC25_C8, and IC25_G4) with 97% or less identity to their closest BLAST relative using the device. These bacterial isolates are currently being further characterized. The ichip device also resulted in the isolation of two strains (IC18_D5 and IC18_D7) identified as belonging to the *Vibrio* genus, extracts from which were subsequently utilized in the EAE of phenolics from *Fucus vesiculosus* and were found to be more efficient in the extraction process than current commercially available enzymes (Figure 2.3). This further demonstrates the utility of the ichip

device as an important method to not only capture previously uncultivable bacteria, but also to recover bacteria with potential biotechnological applications (Berdy et al., 2017; Kealey et al., 2017).

Phylum-level analysis revealed that the structure of both the cultivable and metagenomic microbial communities found on the intact seaweed differed from that of the decaying macroalga, suggesting that the decay process plays a role in altering the algal associated microbial populations. However, these results should be interpreted with caution as our experiments were not conducted in replicates. Similar differences in the microbial community profiles associated with healthy and weakened bleached macroalgae have also been previously reported (Marzinelli et al., 2015). Although a causal link between such differences and the host condition has not been clearly established, it is known that host stress, such as bleaching and decay-related disruptions to the composition and abundance of its associated microbial consortium, can have detrimental effects on the host, causing diseases; for example, due to interferences with the seaweed–bacteria interactions that support algal development and host defense (Rosenberg et al., 2007; Singh and Reddy, 2014). Chun et al., (2017) suggest that microcosms which emerge as a result of the algal decay process may explain the differences in the bacterial populations associated with healthy and decaying algae. Decaying *Cladophora* samples have, for example, been shown to produce low oxygen and pH environments with increased ammonium-nitrogen levels. Subsequently, structural shifts in the microbial community towards bacterial groups better suited to thrive under such conditions were observed (Chun et al., 2017). While the succession of oxygen concentration, pH and nutrient levels during the decay period was not monitored in this study, the structural shifts observed in the microbial communities with decay may be attributed to changes in the composition of the closed microcosm within the shake flasks.

Screening the cultivable surface microbiota of both the intact and decaying seaweed for the production of algal cell wall polysaccharide degrading enzymes revealed a number of MAPD bacteria. Bacteria belonging to the genus *Bacillus* which represented the major producers (>80%) of these hydrolytic enzymes were not identified in the bacterial population associated with *A. nodosum* in its healthy state but represented up to 10% of the surface microbiota communities isolated during the algal decay (Figure S2.1; Supplementary information). This marked difference in the composition and abundance of the microbial communities associated with the seaweed during its different physiological states (intact and decaying), mainly characterized by the emergence in the members of the enzymatically active *Bacillus* and *Vibrio* groups supports the hypothesis that nutrient limiting conditions such as algal decay are likely to promote the proliferation of MAPD producing bacteria (Martin et al., 2015). However, while the number of MAPD isolates was not observed to steadily increase during decay as might be expected due to the weakened state of the seaweed, the few enzymatically active strains that we did identify during decay, such as the *Bacillus* and *Vibrio* species were efficient producers of the MAPD enzymes for which we tested (Table S2.1; Supplementary information).

Microorganisms are well-known to exhibit mutualism such that one or more individuals within a microbial population can gain from the collective characteristics expressed by its neighbors without expressing the trait itself (Egan et al., 2013; Hibbing et al., 2010; Thomas et al., 2008). Such phenotypically deficient bacteria may however possess the metabolic capability necessary to utilize nutrients provided by other members of the community (Thomas et al., 2008). A lack in the increase in the expected numbers of MAPD bacteria that we observed during the decay experiments may thus be explained by the efficiency of the less abundant enzymatically active strains who may be compensating for the inactivity of the dominant species by creating a pool of available nutrients, thereby supporting the overall bacterial

consortia present within the microcosm in the growth flasks, to which no nutrients had been added.

Finally, we assessed the ability of enzymatic bacterial supernatant (EBS) from a selected group of enzyme-active strains; IC18_D7, IC18_D5 and ANT_0_A6 with similarity to *Vibrio anguillarum* X0906, *Vibrio oceanisediminis* S37, and *Winogradskyella sp.* MGE_SAT_697, respectively, in the enzyme-assisted extraction of phenolics from *Fucus vesiculosus*. These enzyme preparations were shown to increase total phenolic content (TPC) extraction yields from *Fucus vesiculosus* by 11–13%, to levels which were greater than the extraction yields obtained using a commercially available xylanase (10%) (Figure 2.3). To our knowledge, this is the first study to report the application of macroalgal-derived bacterial culture extracellular supernatants in the enzyme-assisted extraction of phenolics from *Fucus vesiculosus.* Thus, it is clear that bacterial populations associated with *A. nodosum* are a good source of algal cell wall polysaccharide degrading enzymes with potential utility in EAE strategies. The isolation of macroalgal associated bacteria is frequently reported in the literature (Chun et al., 2017; Martin et al., 2015), with isolates being developed for use in various biotechnological applications, such as novel carrageenanases from *Flavobacteria* and γ-*Proteobacteria* isolated from *Ascophyllum nodosum* (Martin et al., 2015) and from *Pseudoalteromonas porphyrae* isolated from decayed seaweed (Liu et al., 2016) for potential biomedical and food applications, together with alginate lyase from *Zobellia galactanivorans* for biomass degradation (Zhu et al., 2017). Our study further demonstrates the potential utility of algal derived bacteria and their potential contribution to EAE based strategies aimed at the production of seaweed extracts for similar types of biotechnological applications.

2.5. Materials and Methods

2.5.1. Sampling

Ascophyllum nodosum samples were obtained in the intertidal zone at Rinville in Galway Bay, Ireland at 53°14′40″ North, 8°58′2″ West in late January, 2016. Approximately 2 kg of seaweed was sampled and packaged in sterile air-tight plastic bags and stored on dry ice at the sampling location. *Ascophyllum* samples were subsequently stored briefly at 4 °C in the laboratory before further analyses.

2.5.2. Experimental Design

Three sets of approximately 450 g of the seaweed were suspended in separate 950 mL sterile artificial seawater (3.33% *w*/*v* synthetic seawater salts Instant Ocean, Aquarium Systems, in distilled water), with each 2 L flask incubated at a different temperature (18 °C, 25 °C, and 30 °C) on a shaking platform at 125 rpm for a six-week period. The incubation flasks were single replicates (n = 1) per temperature treatment. Three separate ichip devices were subsequently inoculated four weeks after the initial incubation into each of the flasks under sterile conditions in a laminar flow hood (BioAir Safeflow 1.2—EuroClone, Pero, Italy), as previously described (Nichols et al., 2010), and removed at the end of the decay period. To inoculate each ichip device, 1 mL suspension from the incubation flask containing the seaweed decaying at one of the three temperatures (18 °C, 25 °C, and 30 °C), with an estimated microbial density of 1.0×10^{12} cells mL^{-1} was diluted appropriately in sterile artificial seawater (3.33% *w*/*v* synthetic seawater salts Instant Ocean, Aquarium Systems, in distilled water) to attain an average of one cell per through-put hole in the ichip central plate (i.e., one cell per µL of inoculum) and suspended in molten 0.5% agar (Sigma Aldrich, Munich, Germany) solution. The cell-agar suspension was poured over the ichip central plate to allow cells that were immobilized within the suspension to be trapped in the small throughput holes on the device as the agar solidified. The device was then assembled and placed in the flask containing the decaying seaweed for another two weeks. Approximately 10 g of the fresh intact macroalga (T_0) was collected before incubation into the artificial seawater and 10 g of the decaying seaweed was collected from

each incubating flask at two-week intervals (weeks 2, 4, and 6) during the decay period. All *Ascophyllum* samples were stored at −20 ˚C for further analyses.

2.5.3. 16S rRNA Gene Amplicon Library Preparation and MiSeq Sequencing

Metagenomic DNA was extracted from approximately 0.5 g of the intact seaweed (T_0) and 0.5 g each of decaying *Ascophyllum nodosum* samples collected at three phases of the decay period (week 2, week 4 and week 6) at 18 ˚C (2_18, 4_18, 6_18), 25 ˚C (2_25, 4_25, 6_25), and at 30 ˚C (2_30, 4_30, 6_30) as previously described (Varela-Álvarez et al., 2006). PCR amplicon libraries were generated using forward (5^0 *TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*CCTACGGGNGGCWGCAG-3^0) and reverse (*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GACTACHVGGGTATCTAATCC-3^0) primers complementary to the V3-V4 16S rRNA gene region (Klindworth et al., 2013) with ligated Illumina adapter overhang sequences in italic text. This primer pair was identified as the most promising pair required for a good representation of bacterial diversity and has been successfully applied in a number of studies on a wide range of environments (Abrahamsson et al., 2012; Herlemann et al., 2011; Klindworth et al., 2013; Logue et al., 2012). PCR amplification was performed under the following conditions: 98 ˚C for 30 s, followed by 30 cycles of denaturation (98 ˚C for 10 s), primer annealing (57 ˚C for 30 s), primer extension (72 ˚C for 30 s), and 72 ˚C for 5 min. PCR amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions and a subsequent reduced-cycle (8 cycles) reaction was performed to further attach unique dual eight-base Nextera XT multiplexing indices and sequencing adapters under similar cycling conditions. Index PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter; Fisher Scientific, Dublin, Ireland) according to the manufacturer's instructions. All PCR reactions from each sample were performed in triplicates to minimize bias, replicate amplicons

were pooled together and sequenced using the Illumina MiSeq platform by Macrogen (Seoul, Korea).

Scythe (v0.994 BETA) (Buffalo, 2014) and Sickle (Joshi and Fass, 2011) programs were used to quality trim raw reads and remove adapter sequences. This service was provided by Macrogen Inc (Seoul, Korea) as part of a next generation sequencing package. Trimmed paired end reads were merged (using the join_paired_reads.py script with the fastq-join method (Aronesty, 2011)) in QIIME version 1.9.1 (QIIME.org) (Caporaso et al., 2010) and processed using standard QIIME version 1.9.1 protocols (http://qiime.org/tutorials/illumina_ overview_tutorial.html). Briefly, a further quality step was applied by excluding reads with a Phred score less than 20 using the split_libraries_fastq.py QIIME script. The USEARCH algorithm (Edgar, 2010) was used to remove chimeras and assign sequences to OTUs based on the SILVA database (version 123) (Max Plank Institute, Bremen, Germany) (Quast et al., 2012) at a threshold of 97% identity. Singletons were identified and filtered from the OTU table and the OTU table was CSS (cumulative sum scaling) normalized (Paulson et al., 2013). The taxonomy identified from the dataset was then represented through bar plots. Species diversity and richness within samples were calculated using alpha and beta diversity analyses (Chao1, Good's coverage, Shannon indices and principle coordinates analysis) using QIIME (version 1.9.1) (http://qiime.org/tutorials/illumina_overview_tutorial.html) scripts (alpha_diversity.py and beta_diversity_through_plots.py) (Caporaso et al., 2010).

2.5.4. Bacterial Isolation from Intact and Decaying **A. nodosum** *Using Maceration Method*

Surface-attached bacteria were isolated from the intact (T_0) and decaying seaweed samples collected at weeks 2, 4 and 6 of the decay period, each at three different temperatures (18 ˚C, 25 ˚C, and 30 ˚C) using the maceration method adapted from (Santavy et al., 1990). Briefly, approximately 0.5 g of the algal sample was cut into small pieces of about 1 cm^2 and suspended in 1 mL of sterile artificial seawater (3.33% *w*/*v* synthetic seawater salts Instant Ocean,

Aquarium Systems, in distilled water) (Atkinson and Bingman, 1997). Serial dilutions of the suspension were plated on SYP-SW agar plates which consisted of soluble starch (Sigma Aldrich, Munich, Germany) 10 g L^{-1}; yeast extract (Sigma Aldrich, Germany) 4 g L^{-1}; peptone (Merck, Germany) 2 g L^{-1}; Instant Ocean (Aquarium Systems) 33.3 g L^{-1}; agar (Sigma Aldrich, Germany) 15 g L^{-1} and incubated at 28 ˚C for 72 h. The culture isolation procedure was conducted aseptically in a laminar flow hood (BioAir Safeflow 1.2—EuroClone, Pero, Italy). Individual colonies were selected and further streaked to isolate pure cultures which were grown at 28 ˚C overnight in SYP-SW medium and maintained in glycerol (20% *w*/*v*) stocks at −80 ˚C.

2.5.5. Bacterial Isolation from Decaying **A. nodosum** *Using ichip Device*

Three separate ichip devices were inoculated into each of the incubating flasks (18 ˚C, 25 ˚C, and 30 ˚C) at week 4 of the decay period and were removed at the end of the decay period (week 6). Macroalgal-associated bacteria from decaying *A. nodosum* were recovered from small throughput holes on the central plate of each ichip device and plated directly onto 96-well plates containing SYP-SW agar and incubated at 28 ˚C for 72 h. Individual colonies were selected and further streaked to isolate pure cultures which were grown at 28 ˚C overnight in SYP-SW medium and maintained in glycerol (20% *w*/*v*) stocks at −80 ˚C.

2.5.6. Taxonomic Identification of **A. nodosum** *Cultivable Surface Microbiota Populations*

Bacterial isolates recovered from both intact and decaying *A. nodosum* samples using both the traditional maceration method and the ichip method were taxonomically identified using 16S rRNA gene sequencing. Genomic DNA was extracted from the bacterial isolates grown overnight at 28 ˚C in SYP-SW medium using a modified Tris-EDTA boiling DNA extraction method (Li et al., 2003). Bacterial 16S rRNA PCR amplification was performed with the universal forward (8F; 5′-AGAGTTTGATCCTGGCTCAG-3′ or 27F; 5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse (1492R; 5′-GGTTACCTTGTTACGACTT-3′) primers (Lane, 1991; Turner et al., 1999) under the following conditions: initial

denaturation (95 ˚C for 30 s), followed by 35 cycles of denaturation (95 ˚C for 1 min), primer annealing (55 ˚C for 1 min), primer extension (72 ˚C for 1 min) and a final primer extension step (72 ˚C for 5 min). PCR products were analyzed by gel electrophoresis on a 1% agarose gel and purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Sanger sequencing was performed on the amplified PCR products by GATC Biotech, (Konstanz, Germany) and Macrogen (Amsterdam, The Netherlands). Low quality 5' and 3' sequence ends were trimmed using FinchTV (http://www.geospiza.com/finchtv) depending on the data set. The BLAST program (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare trimmed sequences against the GenBank database and closest relatives to the bacterial isolates were identified. 16S rRNA gene sequences were checked for chimeras using USEARCH algorithm (Edgar, 2010) and the data sets were de-replicated using the Fastgroup database (Seguritan and Rohwer, 2001) and Avalanche NextGen Workbench version 2.30 (http://www.visualbioinformatics.com/html/)(bioinformatics.org) with a 99% cut-off value. Sequence alignment and phylogenetic tree construction were performed with MEGA (version 7) (Penn State University, PA, USA) (Kumar et al., 2016). The evolutionary history was inferred using the Neighbor-joining method (Saitou and Nei, 1987).

2.5.7. Enzyme Screens

Bacterial isolates obtained from both intact *A. nodosum* and the decaying seaweed, using both the maceration method and the ichip device, were screened for the production of macroalgal cell wall degrading enzymes including pectinase, hydroxyethyl cellulase, and enzymes involved in lichenin degradation. Bacterial isolates were grown at 28 ˚C for 72 h on LB gellan gum (Sigma Aldrich, Munich, Germany) plates supplemented with the appropriate substrate, at a concentration of 0.2% (*w*/*v*) for pectin (Sigma Aldrich, Germany), 0.5% (*w*/*v*) for hydroxyethyl cellulose (Sigma Aldrich, Germany) and 0.05% (*w*/*v*) for lichenin (Megazyme).

Enzymatic activities on lichenin and HE-cellulose were indicated by a surrounding zone of clearance upon flooding with Congo red solution (0.1% *w*/*v* Congo red in 20% *v*/*v* ethanol) for 30 min and wash with 1M NaCl for 5 min (Walter et al., 2005; Wolf et al., 1995) while pectin supplemented plates were flooded with Lugol's iodine solution (Soares et al., 1999).

2.5.8. Enzyme-Assisted Extraction

Entire specimens of *Fucus vesiculosus* (2–3 kg fresh weight) were collected at low tide on 6 July 2016 at Finavarra, Co., Clare, Ireland (53° 08′ 59″ North–9° 08′ 09″ West). In the laboratory, on the day of collection, algal biomass was cleaned of epiphytes and rinsed in distilled water to remove excess salt. Samples were patted dry with tissue paper and stored at −20 °C. Then, the biomass was freeze-dried in a Labconco Freezone® freeze-dryer system (Labconco Corporation, Kansas City, MO 64132-2696, USA). Dried biomass was ground using a coffee-grinder and sieved to produce two types of powder, Ps < 0.5 mm and 0.5 < Ps < 2.5 mm), prior to subsequent enzymatic-assisted extraction (EAE) of total phenolic compounds. Three commercially available enzymes; cellulase (from *Aspergillus* sp., Sigma Aldrich, ≥1000 U/g), xylanase (from *Trichoderma* sp., Megazyme, 2.86 U/mL) and protease (from *Bacillus licheniniformis*, Sigma Aldrich, ≥2.4 U/g) were used for the hydrolysis of the seaweed. The potential of three bacterial strains (IC18_D7, IC18_D5 and ANT_0_A6 with ≥ 98% sequence similarity to *Vibrio anguillarum* X0906, *Vibrio oceanisediminis* S37 and *Winogradskyella* sp. MGE_SAT_697, respectively) isolated from *A. nodosum* and shown to be producers of algal cell wall degrading enzymes in this study was also tested. Bacterial isolates were grown overnight at 28 °C in SYP-SW medium and equal volumes of supernatants from the overnight cultures obtained by centrifugation at 4300× *g* for 10 min were used. Three sets of approximately 4 g dry weight of the crushed algae was incubated at 50 °C for 24 h on a shaking platform (185 rpm) with sodium acetate buffer (100 mL; 0.1 M; pH 5.2), each with 100 µL of one of the three commercial enzymes. Another set of the algal biomass was also

incubated at 28 °C with a mixture of culture supernatants obtained from the bacterial isolates (enzymatic bacterial supernatants, EBS) to a final volume of 100 µL. All experiments were performed in triplicate and control experiments without the addition of either commercial enzymes or culture supernatants (EBS) were also conducted under the same conditions. The hydrolysate mixture from each experimental set was centrifuged at 4300× *g* for 10 min at 4 °C to eliminate the algal debris from the extract. The different extracts produced were freeze dried, weighed, and stored at −80 °C until further analysis for total phenolics.

2.5.9. Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of the *F. vesiculosus* crude extracts was determined using a slightly modified version of the Folin–Ciocalteu assay (Singleton and Rossi, 1965) as described by (Kenny et al., 2013). A known amount of crude extract was re-suspended in methanol to a concentration of 1 mg.mL^{-1}. 100 µL of each crude extract was placed in a 1.5 mL Eppendorf tube along with 100 µL of methanol, 100 µL of Folin–Ciocalteu reagent (2N) and 700 µL of 20% sodium carbonate, to a final volume of 1 mL. Samples were vortexed and immediately afterwards placed in darkness to incubate for 20 min at room temperature. Samples were then centrifuged at 4300× *g* for 3 min before measuring the absorbance of the supernatant at 735 nm using a Cary UV50 Spectrophotometer and CaryWIN software (Varian Inc., Palo Alto, CA 94304, USA). A sample treated according to the same protocol, but where 100 µL methanol instead of 100 µL of crude extract (1 mg·mL^{-1}) was added, was used as a blank. Phloroglucinol was used as the external standard and a calibration curve was performed by serial dilution of a 2 mg mL^{-1} stock solution (10, 20, 50, 80, 120, 160 µg mL^{-1}). Total phenolic content (TPC) was expressed as milligram of phloroglucinol equivalents (PE) per gram of dry weight extract (mg PE g^{-1} DWE) or per gram of dry weight biomass (mg PE g^{-1} DWB) (Kenny et al., 2015). TPC quantification was performed in triplicate for each crude extract. The yield of extraction was calculated after exhaustive solid-liquid extraction (i.e., three successive extraction) of the

total phenolics of 50 mg of *F. vesiculosus* freeze-dried ground biomass (Ps < 0.5 mm) using 80% methanol.

2.5.10. Accession Numbers

The metagenomic sequencing data (raw reads) was deposited in the European Nucleotide Archive (ENA) under the accession numbers ERR2608102 -ERR2608111. The 16S rRNA gene sequences for the bacterial isolates were deposited in GenBank under the accession numbers KY224981–KY225289, KY327837, KY327838, MG693225–MG693716, MG760723–MG760725, and MK480287–MK480325. Bacterial isolates IC18_D7 and ANT_0_A6 with enzymatic activities and applicability in EAE approach were deposited in DSMZ culture collection bank under the accession numbers DSM 107285 and DSM 107318, respectively.

2.5.11. Statistical Analysis

Prior to performing statistical analyses on data obtained by enzyme-assisted extraction (EAE) of total phenolics from *F. vesiculosus*, tests of normality were carried out with the Kolmogorov–Smirnov test for normal distributions and Levene's test for homogeneity of variance. A one-way ANOVA and post hoc Tukey's pairwise test was performed to assess significant differences ($p < 0.05$) between commercial enzymes and control (50 °C); and a *t*-test was applied to assess significance differences ($p < 0.05$) between EBS and control (28 °C). All data treatments and statistical analyses were performed using IBM SPSS Statistics V22.0 (IBM Corporation, Armonk, NY, USA).

2.6. Conclusion

In conclusion, we have demonstrated, using both metagenomic and culture based approaches, that changes occur in the composition and abundance of *A. nodosum*-associated epibiotic communities which are both time and temperature dependent and that the microflora of *A. nodosum* is composed of diverse and complex bacterial communities which produce a wide

range of hydrolytic enzymes, some of which may be useful in future EAE based strategies in the agricultural, food, cosmeceutical, and pharmaceutical sectors.

Supplementary Materials:

Table S2.1: Table showing enzymatic activities, Figure S2.1: *Ascophyllum* cultivable surface microbiota at genus classification level, Figures S2.2–S2.6; Phylogenetic trees of intact and decaying *A. nodosum* associated microbial communities.

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Chapter 3: Microbial population changes in decaying *Laminaria digitata* result in an enrichment in bacteria with enzymes capable of algal cell wall degradation.

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3.1 Abstract

Macroalgae are of great interest in both industry and agriculture as they contain many potentially very useful bioactive compounds. Several environmental and non-environmental factors can influence the composition and abundance of epibacterial communities associated with seaweeds. In addition to seasonal variations, the physiological (fresh or decayed) state of the macroalgal surface, has also been found to play a significant role in the structure of the associated microbial communities. Marine microorganisms associated with algae are also known to produce diverse hydrolytic enzymes such as polysaccharidases which improve algal nutrient uptake and development while also contributing to algal defence from potential grazers. These diverse and complex bacterial communities thus represent a potential source of novel enzymes with biotechnological applications that could include enzyme-assisted extraction (EAE) strategies resulting in the improvement of the yields of algal components with cosmeceutical, functional food, and nutraceutical and biopharmaceutical applications. In this study, changes in the microbial communities associated with Irish brown seaweed *Laminaria digitata* incubated in artificial seawater at three different temperatures (20 °C, 25 °C and 30 °C) for four weeks were examined using both culture dependent and culture independent, metagenomic based approaches. The cultivable surface microbiota of the seaweed was also screened for the production of algal cell wall degrading enzymes. The epibacterial communities evolved during decay to include functionally active bacteria, with more than 50% of the population derived from the decaying seaweed being found to produce algal cell wall degrading bacteria.

3.2 Introduction

There is an increased interest in the use of biomass from marine macroalgae (seaweeds) as potential sustainable feedstock for the production of animal feed ingredients (Overland et al., 2017) and in the production of biofuels and bioenergy; where it is regarded as a "third generation biomass" (Ghadiryanfar et al., 2016). This is reflected in the fact that the commercial seaweed market which was valued at USD 13.9 billion in 2017, is projected to reach $22.32 billion by 2025 (Research, 2017); together with significant increases in the cultivation of seaweed, from 6.5 million tons in 2001 with a value of US $ 1,768M to 15.8 million tons in 2010 with the value of US $ 4,143M, of which 43% are brown seaweeds (Ghadiryanfar et al., 2016). Brown seaweeds such as *Laminaria* species are a rich source of polysaccharides such as laminarin and fucoidan which have a variety of functional food and animal feed applications, as well as alginates which in addition to having applications in the food and beverage industry also have biomedical and bioengineering applications, primarily due to their biocompatibility, biodegradability together with possessing biological and pharmacological activities (Christensen, 2011; Lee and Mooney, 2012; Szekalska et al., 2016).

Laminaria digitata (Hudson) J.V. Lamouroux is a brown seaweed which is commonly found in the low water shore areas of the North European and Eastern North American coastlines. Extracts from *L. digitata* containing polysaccharides such as laminarin and fucoidan have been reported as an alternative to in-feed antibiotics in pigs and as an alternative to therapeutic doses of zinc oxide in pig diets (Lynch et al., 2010; O'shea et al., 2014; Walsh et al., 2013). Improvements in overall pig health have been reported following the supplementation of the diet of weanling pigs with laminarin and fucoidan extracts from *L. digitata* (Walsh et al., 2013). While the feeding of *L. digitata* extracts containing fucoidan and laminarin has been reported to improve the quality and shelf life of pork and reduce lipid oxidation in muscle tissue (Moroney et al., 2013).

Macroalgae are also gaining increased attention with respect to their potential applications in the production of biofuels and bioenergy (Ghadiryanfar et al., 2016). They are attractive as feedstocks for sustainable bioenergy systems due to their higher growth rates when compared to terrestrial-based energy crops (Choi et al., 2014; Tabassum et al., 2018). In addition, the fact that they lack lignin increases the efficiency of anaerobic digestion (AD) processes in which they are involved (Fasahati et al., 2017). Recent work on the AD of various brown seaweeds has shown that *L. digitata* possesses the highest biomethane potential when compared to other Irish seaweeds such as *Laminaria hyperborean*, *Saccharina latissima* and *Saccorhiza polyschides* (Tabassum et al., 2018). *L. digitata* also has a relatively low ash (25.7 % dw) and nitrogen content (0.9% dw) and a favourable composition when compared to some other seaweeds that are being considered as potential feedstocks for biofuels (Allen et al., 2015; Milledge and Harvey, 2016). Furthermore, it contains high concentrations of mannitol, reported to be above 30% on a weight basis (Adams et al., 2011) which has recently been targeted as a substrate for the production of bioethanol by fermentative thermophilic *Clostridia* following extraction of the mannitol from the alga using weak acids (Chades et al., 2018).

Whether extracts from *L. digitata* are to be used for animal/fish feed, food, biomedical or biofuels and bioenergy applications, it is clear that efficient cost effective and ideally environmentally friendly extraction procedures of these useful extracts are required. With respect to the extraction of polysaccharide fractions from macroalgae, methods that are currently employed typically include a solvent extraction step followed by extraction technologies such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) or enzyme-assisted extraction (EAE) (Garcia-Vaquero et al., 2017). The latter extraction technology is gaining traction as an alternative to conventional solvent based approaches, given the high catalytic efficiency and specificity involved, together with the mild reactive conditions (Kulshreshtha et al., 2015). Additional benefits include increased efficiency

and more environmentally friendly processes (Kadam et al., 2013). The more widespread use of EAE based approaches is being hindered to some extent by the need for well-defined enzymes or enzyme mixtures to facilitate a good recovery of the compound of interest or liberation of the fermentation substrate for the production of biofuels (Jeon et al., 2011). Enzymes which are typically used in EAE include amyloglucosidases, agarases, alcalases, carragenanases, and xylanases, together with cellulase protamex, kojizyme, neutrase and viscozyme (Garcia-Vaquero et al., 2017).

Epiphytic bacteria which colonize the surface of macroalgae should be a good source of these types of enzymes for use in extraction processes as they are known to produce a diverse repertoire of hydrolytic enzymes such as polysaccharide hydrolases, which typically function to aid in algal nutrient uptake and defense from potential grazers (Egan et al., 2013). A number of bacterial species, commonly referred to as macroalgal polysaccharide degrading (MAPD) bacteria, which possess the ability to enzymatically catabolise algal cell wall constituents (Zhu et al., 2016) have been isolated from macroalgae. The well-studied *Zobellia galactanivorans* isolated from the red alga *Delesseria sanguinea* which is known to possess a complex enzymatic system involving Carbohydrate Active enZymes (CAZymes) to degrade polysaccharides is the best studied system to date (Thomas et al., 2017). While *Algibacter alginolytica* isolated from *Laminaria japonica* also possesses numerous glycoside hydrolases and polysaccharide lyases (Sun et al., 2016). Bacteria with alginate lyase activity have also been isolated from *Laminaria* in the Arctic with *Psychrobacter*, *Psychromonas* and *Polibacter* being the predominant producing groups (Dong et al., 2012).

In a bid to isolate bacteria with potentially improved capabilities of algal cell wall degradation which could be applicable in a number of biotechnological technologies, particularly enzyme-assisted extraction strategies, this work focuses on the controlled decay of the brown seaweed *Laminaria digitata* as a habitat for such MAPD bacteria. The macroalgal decay process has

been reported as a physiological condition which promotes the proliferation of MAPD bacteria (Martin et al., 2015). These bacteria are well known to be involved in the recycling of algal biomass and in global carbon cycling (Martin et al., 2015), hence, the proportion of MAPD bacteria on the surface of seaweeds can be expected to increase in weakened or unhealthy algae (Martin et al., 2015). Recent studies on *Cladophora* and *Ascophyllum nodosum* also indicate that algal decay results in significant changes in the structure and composition of seaweed-associated microbial communities (Chun et al., 2017; Ihua et al., 2019). Furthermore, in previous work, we have reported the use of *Ascophyllum*-derived bacteria with efficient algal cell wall hydrolytic activities have been successfully employed in the enzyme-assisted extraction of phenolics from the brown alga *Fucus vesiculosus* (Ihua et al., 2019). In this study, the microbial populations associated with both intact and decaying *L. digitata* was monitored using both metagenomic and culture based approaches, which included the novel ichip device which has been reported to aid in the isolation of previously uncultivable bacteria (Berdy et al., 2017; Kealey et al., 2017; Nichols et al., 2010), and the algal cell wall degrading capabilities of the cultivable surface microbiota was also examined. The associated epibacterial communities of *L. digitata* were found to change distinctly upon decay, to include a range of *Bacillus* and *Pseudoalteromonas* species, which produced a number of algal cell wall degrading enzymes such as lichenases, pectinases and hydroxyethyl cellulases.

3.3 Materials and Methods

3.3.1 Sampling

Laminaria digitata meristem samples were obtained from the intertidal zone at Spiddal in Co. Galway, Ireland at 53º 24' 41'' North, 9º 30' 58'' West in February, 2016. Approximately 150 g of the seaweed was sampled and packaged in sterile air-tight plastic bags and stored on dry

ice at the sampling location. The seaweed samples were subsequently briefly stored at -20 ºC in the laboratory before further analyses.

3.3.2 Experimental design

Approximately 30 g of the seaweed was suspended in 500 ml sterile artificial seawater (3.33% *w/v* synthetic seawater salts Instant Ocean, Aquarium Systems, in distilled water) (Atkinson and Bingman, 1997) in a 2,000 ml incubation flask. The algal decay experiment was set up with three sets of seaweed samples, with each set incubated at a different temperature (20 °C, 25 °C and 30 °C) on a shaking platform at 125 rpm and allowed to decay for four weeks. The incubation flasks were single replicates (n = 1) per temperature treatment. Three separate iChip devices were inoculated into each of the flasks under sterile conditions in a laminar flow hood (BioAir Safeflow 1.2—EuroClone, Pero, Italy), as previously described (Nichols et al., 2010) two weeks after the initial incubation. Briefly, 1 mL suspension from each incubation flask which contained the decaying seaweed was diluted appropriately in sterile artificial seawater to achieve an average of one cell per through-put hole in the ichip central plate (i.e., one cell per µL of inoculum) and suspended in molten 0.5% agar (Sigma Aldrich, Munich, Germany) solution. The cell-agar suspension was poured over the ichip central plate to allow cells that were immobilized within the suspension to be trapped in the small throughput holes on the device as the agar solidified. The ichip device was assembled and introduced into the respective incubation flasks until the end of the decay process (week 4). Approximately 3 g of the intact seaweed (LDT0) was collected prior to the induced decay and 10 ml suspension from the decaying samples were collected from each incubating flask weekly during the decay period. All *Laminaria* samples were stored at −20 ˚C for further analyses.

3.3.3 16S rRNA gene amplicon library preparation and MiSeq sequencing

The metagenomic communities of both intact and decaying *Laminaria digitata* samples which were collected on a weekly basis (week 1 to week 4) from the three different temperature incubation flasks (20ºC, 25°C and 30°C) were analyzed using 16S rRNA metagenomic sequencing targeting the V3-V4 16S rRNA gene region. Metagenomic DNA was extracted from approximately 0.5 g of the intact seaweed (LDT0) and 0.5 ml suspension each of decaying *Laminaria digitata* samples as previously described (Varela-Álvarez et al., 2006). PCR amplicon libraries were generated using forward (5'-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*CCTACGGGNGGCWGCAG -3') and reverse (5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GACTACHVGGGTATCTAATCC -3') (Klindworth et al., 2013) with ligated Illumina adapter overhang sequences in italic text. Previous studies have identified this set of primers as the most promising pair required to achieve a good representation of bacterial diversity and their use has been reported in a wide range of environmental studies (Abrahamsson et al., 2012; Klindworth et al., 2013; Logue et al., 2012). The PCR amplification was performed under the following conditions: 98°C for 30 s, 30 cycles of denaturation (98°C for 10 s), primer annealing (57°C for 30 s), primer extension (72°C for 30 s) and 72°C for 5 min. The amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter, Fisher Scientific, Dublin, Ireland) according to the manufacturer's instructions and unique dual eight-base Nextera XT multiplexing indices and sequencing adapters were subsequently attached to the purified amplicons in a reduced-cycle (8 cycles) PCR reaction under similar cycling conditions. Indexed PCR amplicons were further purified using Agencourt AMPure XP beads (Beckman Coulter, Fisher Scientific, Dublin, Ireland) according to the manufacturer's instructions. All PCR reactions from each sample

were performed in triplicates, pooled together and sequenced using the Illumina Miseq platform by Macrogen (Seoul, Korea).

3.3.4 Taxonomic assignment of OTUs (operational taxonomic unit)

Raw reads generated from the MiSeq sequencing were trimmed using Scythe (v0.994 BETA) (Buffalo, 2014) and Sickle (Joshi and Fass, 2011) programs to exclude adapter sequences as a supplementary sequencing service provided by Macrogen Inc (Seoul, Korea). The trimmed paired end reads were merged in QIIME version 1.9.1 (QIIME.org) (Caporaso et al., 2010), using the join_paired_reads.py script with the fastq-join method (Aronesty, 2011) and processed according to standard QIIME version 1.9.1 protocols (http://qiime.org/tutorials/illumina_ overview_tutorial.html). The reads were applied to a quality step using the split_libraries_fastq.py QIIME script which excluded reads with a Phred score less than 20. Chimeric sequences were also identified and removed from the dataset using the USEARCH algorithm (Edgar, 2010). Qualified sequence reads were further assigned to taxonomy based on the SILVA database (version 123) (Max Plank Institute, Bremen, Germany) (Quast et al., 2012) at a threshold of 97% identity using the USEARCH algorithm (Edgar, 2010). The resultant table was filtered of singletons, CSS (cumulative sum scaling) normalized (Paulson et al., 2013) and the taxa present were then represented through bar plots. Finally , alpha and beta diversity analyses (Chao1, Good's coverage, Shannon indices and principle coordinates analysis) were performed using QIIME (version 1.9.1) (http://qiime.org/tutorials/illumina_overview_tutorial.html) scripts (alpha_diversity.py and beta_diversity_through_plots.py) (Caporaso et al., 2010) to observe the species diversity and richness within the algal samples.

3.3.5 Bacterial isolation from intact L. digitata

The cultivable surface microbiota of intact *L. digitata* was isolated using the conventional maceration method (Santavy et al., 1990). Approximately 0.5 g of the seaweed was finely cut

into small pieces of about 1 cm^2 and suspended in 1 ml of sterile artificial seawater (Atkinson and Bingman, 1997) under aseptic conditions in a laminar flow hood (BioAir Safeflow 1.2—EuroClone, Pero, Italy). The seaweed suspension was serially diluted in sterile artificial seawater and plated on SYP-SW agar plates consisting of 10 g l^{-1} soluble starch (Sigma Aldrich, Munich, Germany); 4 g l^{-1} yeast extract (Sigma Aldrich, Munich, Germany); 2 g l^{-1} peptone (Merck, Germany); 33.3 g l^{-1} Instant Ocean (Aquarium Systems); 15 g l^{-1} agar (Sigma Aldrich, Munich, Germany) and incubated at 28 ^{0}C for 72 hours. Individual colonies were selected and further streaked to isolate pure cultures which were grown at 28 °C overnight in SYP-SW medium and maintained in glycerol (20% *w/v*) stocks at -80 ^{0}C.

3.3.6 Bacterial Isolation from Decaying **L. digitata** *Using ichip Device*

The ichip devices which were inoculated into each of the incubating flasks containing the seaweed decaying at 20 °C, 25 °C and 30 °C were removed at the end of the decay period (week 4). Bacteria associated with decaying *Laminaria digitata* were obtained from agar plugs within small throughput holes on the central plate of each ichip device and plated on SYP-SW agar plates and incubated at 28 ˚C for 72 h. Individual colonies were selected and further streaked to isolate pure cultures which were grown at 28 ˚C overnight in SYP-SW medium and maintained in glycerol (20% *w/v*) stocks at −80 ˚C.

3.3.7 Taxonomic Identification of **L. digitata** *Cultivable Surface Microbiota Populations*

All *Laminaria digitata* associated surface attached bacteria were taxonomically identified using 16S rRNA gene sequencing of the V3-V4 gene regions. The bacterial isolates were grown overnight at 28 ˚C in SYP-SW medium and genomic DNA was extracted using a modified Tris-EDTA boiling DNA extraction method (Li et al., 2003). PCR amplification was performed with the universal forward (27F; 5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (1492R; 5'-GGTTACCTTGTTACGACTT-3') primers (Lane, 1991; Turner et al.,

1999) under the following conditions: initial denaturation (95 ˚C for 30 s), followed by 35 cycles of denaturation (95 ˚C for 1 min), primer annealing (55 ˚C for 1 min), primer extension (72 ˚C for 1 min) and a final primer extension step (72 ˚C for 5 min). PCR amplicons were analyzed by gel electrophoresis on a 1% agarose gel, purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and sequenced by Eurofins Genomics (Ebersberg, Germany).

The sequence dataset was trimmed using FinchTV (http://www.geospiza.com/finchtv) to remove low quality 5' and 3' ends. Trimmed reads were compared to the GenBank database and closest relatives to the bacterial isolates were identified using the NCBI BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Chimeric sequences were identified using USEARCH algorithm (Edgar, 2010) and removed and the data sets were de-replicated using Avalanche NextGen Workbench version 2.30 (http://www.visualbioinformatics.com/html/) with a 99% cut-off value. A representative phylogenetic tree was constructed using MEGA (version 7) (Penn State University, PA, USA) (Kumar et al., 2016) using the Neighbor-joining method (Saitou and Nei, 1987).

3.3.8 Enzyme screens

The total cultivable epibacterial population isolated from both intact and decaying *Laminaria digitata* in this study was screened for the production of algal cell wall degrading enzymes, including lichenase, pectinase and hydroxyethyl cellulase. The bacterial isolates were grown at 28 ˚C for 72 h on LB gellan gum (Sigma Aldrich, Munich, Germany) plates supplemented with the appropriate substrate, at a concentration of 0.05% (*w*/*v*) for lichenin (Megazyme, Wicklow, Ireland), 0.2% (*w*/*v*) for pectin (Sigma Aldrich, Germany) and 0.5% (*w*/*v*) for hydroxyethyl cellulose (Sigma Aldrich, Germany). Pectin containing plates were flooded with Lugol's iodine solution to reveal a zone of clearance indicative of positive enzyme activity

(Soares et al., 1999), whereas lichenase and HE-cellulase activities were indicated by a surrounding zone of clearance upon flooding with Congo red solution (0.1% *w*/*v* Congo red in 20% *v*/*v* ethanol) for 30 min and wash with 1M NaCl for 5 min (Walter et al., 2005; Wolf et al., 1995).

3.3.9 Accession numbers

The metagenomic sequencing data (raw reads) was deposited in the European Nucleotide Archive (ENA) under the accession numbers ERX3552765-ERX3552777. The 16S rRNA gene sequences for the bacterial isolates were also deposited in GenBank under the accession numbers MK934994 - MK935143.

3.4 Results

3.4.1 MiSeq Sequencing and Data Processing

Using Illumina MiSeq sequencing which targeted the V3-V4 16S rRNA gene region, the microbial communities of *Laminaria digitata* samples were analysed based on their physiological state; intact (LDT0) or decayed at 20 °C, 25 °C and 30 °C. The next generation sequencing produced a total of 8,783,590 raw reads which when quality filtered produced 3,545,422 reads with an average length of 456 bp and were downstream processed using the QIIME version 1.9.1 (http://qiime.org/tutorials/illumina_overview_tutorial. html) workflow (Caporaso et al., 2010). Table 3.1 shows the number of reads obtained after quality filtering and the number of operational taxonomic units (OTUs) identified in each sample. The species richness and diversity indices of the microbial communities are also shown. Approximately 5% of the OTUs identified in intact *Laminaria digitata* samples and up to 20% of the decaying seaweed were not assigned to taxonomy. These unclassified OTUs possibly represent bacterial

populations not present in the SILVA version 132 database (Quast et al., 2012) which was used for taxonomy assignment, or are as yet unknown.

Table 3.1: Observed OTUs together with species richness and alpha diversity estimates of *Laminaria digitata*-associated metagenomic communities obtained using MiSeq sequencing of the 16S rRNA gene from the intact macroalga (LDT0) and each of decaying *Laminaria* samples collected at four weeks of the decay period (week 1, week 2, week 3 and week 4) at 20 ˚C (LDT120, LDT220, LDT320, LDT420), 25 ˚C (LDT125, LDT225, LDT325, LDT425), and at 30 ˚C (LDT130, LDT230, LDT330, LDT430).

Decay Period	Sample	No. of Reads after Quality Filtering	No. of OTUs (at 97% Sequence Identity)	Chao1 Richness	Shannon Index
Week 0 intact seaweed	LDT0	384,328	600	651.0	8.9
Week 1	LDT120	429444	1649	3020.2	10.3
	LDT125	331756	1278	2760.0	9.9
	LDT130	337717	1621	3225.5	10.3
Week 2	LDT220	237349	842	1580.6	9.3
	LDT225	304818	1238	2456.2	9.9
	LDT230	262465	1653	3324.0	10.3
Week 3	LDT320	278877	1415	3415.7	10.1
	LDT325	275098	1393	3100.0	10.1
	LDT330	235814	1875	4987.0	10.5
Week 4	LDT420	199596	1537	3310.7	10.2
	LDT425	57162	588	1171.0	8.9
	LDT430	210998	1827	4207.7	10.5

3.4.2 Intact L. digitata *associated metagenomic communities*

The metagenomic communities associated with intact *L. digitata* were found to comprise of bacteria belonging to six different bacterial phyla including *Proteobacteria, Firmicutes, Planctomycetes, Actinobacteria, Bacteroidetes* and *Chloroflexi* (Figure 3.1a). The phylum *Proteobacteria* was found to dominate the microbial population, with up to 65% relative

abundance. Members of the families *Pseudoalteromonadaceae*, *Psychromonadaceae*, *Rhodobacteraceae*, *Pseudomonadaceae*, *Burkholderiaceae*, *Sphingomonadaceae* and *Halomonadaceae* which belong to the phylum *Proteobacteria* were identified (Figure 3.2a). Other bacterial families such as *Bacillaceae*, *Paenibacillaceae*, *Staphylococcaceae* within the phylum *Firmicutes* and *Micrococcaceae* and *Pirellulaceae* which belong to *Actinobacteria* and *Planctomycetes* respectively were also identified in the sequence reads derived from the intact seaweed. Only a few OTUs (600) were identified and this was also reflected in the richness and diversity indices (Table 3.1).

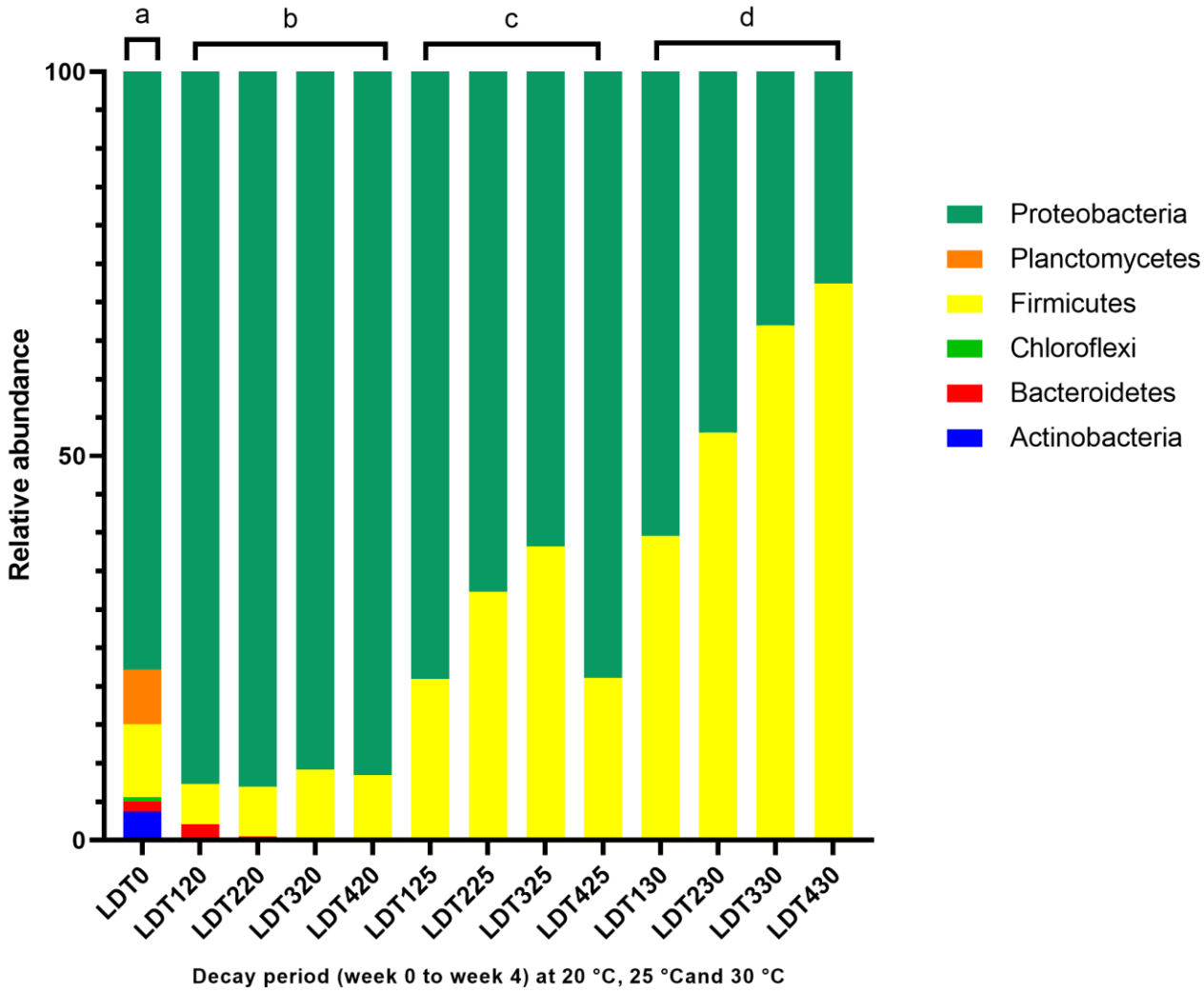

Figure 3.1: Relative abundances of bacterial phyla associated with (a) intact (LDT0) and decaying *Laminaria digitata* at four weeks of decay (week 1, week 2, week 3 and week 4) at (b) 20 ˚C (LDT120, LDT220, LDT320, LDT420), (c) 25 ˚C (LDT125, LDT225, LDT325, LDT425), and (c) 30 ˚C (LDT130, LDT230, LDT330, LDT430) obtained from metagenomic 16S rRNA gene sequencing. The relative distribution of phyla in each group is represented as a percentage.

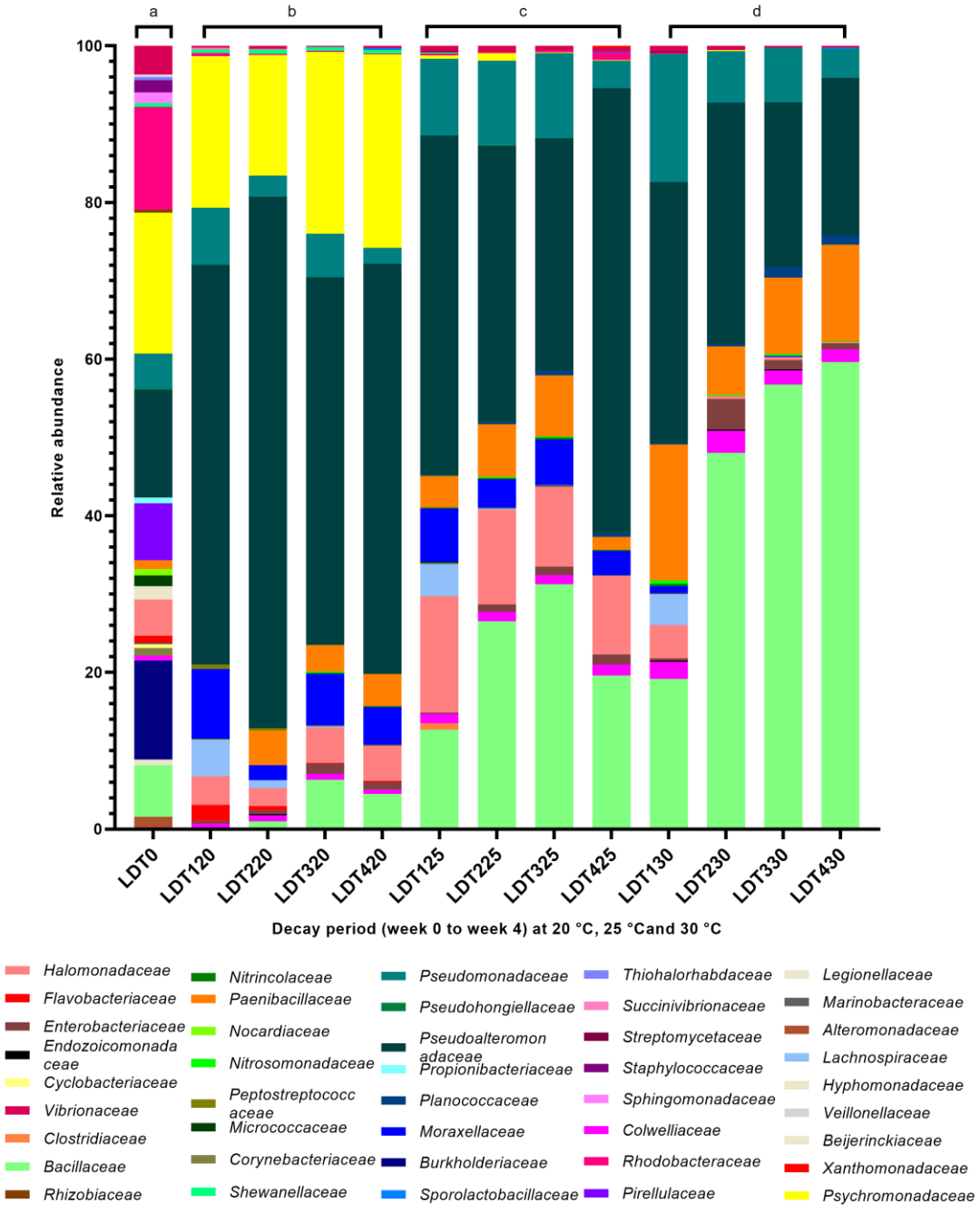

Figure 3.2: Relative abundances of bacterial families associated with (a) intact (LDT0) and decaying *Laminaria digitata* at four weeks of decay (week 1, week 2, week 3 and week 4) at (b) 20 ˚C (LDT120, LDT220, LDT320, LDT420), (c) 25 ˚C (LDT125, LDT225, LDT325, LDT425), and (c) 30 ˚C (LDT130, LDT230, LDT330, LDT430) obtained from metagenomic 16S rRNA gene sequencing. The relative distribution of phyla in each group is represented as a percentage.

3.4.3 Microbial population changes in L. digitata *decaying at 20 °C*

Distinct differences between the microbial populations associated with the seaweed prior to decay (described in session 3.4.2) and during decay at 20 °C over a period of four weeks were observed. In particular, the phyla *Planctomycetes* and *Chloroflexi* were not identified in the decaying seaweed while *Actinobacteria* which was previously present in the intact seaweed at 3% relative abundance was found to have decreased to 0.1% in week 1 (Figure 3.1b). Within the *Proteobacteria* group present, bacteria belonging to the family *Rhodobacteraceae* which was identified at 10% relative abundance prior to decay was observed to have declined to less than 0.5% within the first week of decay and *Burkholderiaceae* (10%; LDT0) was not found during decay. On the other hand, some families such as *Moraxellaceae* and *Lachnospiraceae* were observed to have emerged within the first week of algal decay at 20 °C. The composition of the metagenomic communities associated with the decaying seaweed remained largely unchanged in subsequent weeks of decay (Figure 3.1a), but the relative abundance of the bacterial groups present varied at different time points. The relative abundance of bacteria classified as *Pseudoalteromonadaceae* was observed to have increased from approximately 11% in the intact seaweed to over 40% at week 1 of the decay period. The presence of this group of bacteria increased further at week 2 (53%) but declined to approximately 37% in the late phase of the algal decay (week 3 and week 4). The proportion of *Bacillaceae* in the

decaying seaweed progressed from being not identified in week 1 to a relative abundance of 3% by week 4. In addition, the levels of *Psychromonadaceae* and *Halomonadaceae* were maintained at around 16% and 3% respectively at week 1 to 18% and 3% respectively at the end of the algal decay period (Figure 3.2).

3.4.4 Microbial population changes in L. digitata *decaying at 25 °C*

During the algal decay at 25 ^{o}C, at the phylum level, *Proteobacteria* dominated the bacterial population (>50% relative abundance), followed by *Firmicutes* (>20%) while *Actinobacteria* was scarcely present (>0.5%) in the data set. The presence of bacteria classified as *Bacillaceae* which belong to the phylum *Firmicutes* was shown to be increasingly abundant during decay, from levels of 9% found at week 1 to 19% and 23% at week 2 and week 3 respectively. However, this group of bacteria experienced a decline in its relative abundance at the end of the decay period (15% at week 4). A similar pattern of increase and decline in relative abundance was also observed in bacteria classified as *Paenibacillaceae* also within the phylum *Firmicutes*. Metagenomic analysis also reveal that as the levels of *Bacillaceae* and *Paenibacillaceae* increased, the relative abundance of *Pseudoalteromonadaceae* in the phylum *Proteobacteria* decreased (Figure 3.2b). *Burkholderiaceae* and *Alteromonadaceae* within the *Proteobacteria* group which were previously present in the intact seaweed were not found to be present during decay at 25 ^{o}C. The seaweed decaying at 25 ^{o}C was marked by the presence of *Halomonadaceae* (7%), whereas *Psychromonadaceae* was identified at low relative abundance (<1%) at this incubating temperature. Furthermore, other bacterial families such as *Lachnospiraceae* and *Moraxellaceae* not identified prior to decay were observed to be present in the decaying seaweed.

3.4.5 Microbial population changes in L. digitata *decaying at 30 °C*

At 30 °C, the microbial populations associated with the decaying seaweed was largely dominated by bacteria which belong to the family *Bacillaceae* from the phylum *Firmicutes*, reaching up to 45% relative abundance in week 4 (Figure 3.2c). In the intact seaweed, this group of bacteria was present at a relative abundance of 5% but steadily increased (13%, 35%, 43% and 45% from week 1 to weeks 2, 3 and 4 respectively) during the period of decay. This increase in the relative abundance of *Bacillaceae* was observed to occur concurrently with a decline in the relative abundance of bacteria identified as *Pseudoalteromonadaceae* in the decaying seaweed. Some families such as *Burkholderiaceae*, *Pirellulaceae*, *Staphylococcaceae*, *Rhodobacteraceae* and *Psychromonadaceae* present in the intact seaweed were either not identified during decay or were found to be present at less than 0.1% relative abundance.

3.4.6 Cultivable Surface Microbiota Community of intact L. digitata

Using the conventional maceration isolation method, the cultivable surface microbiota of intact *Laminaria digitata* was assessed. Up to 23 bacterial isolates cultured from the intact seaweed were taxonomically classified following 16S rDNA sequence analysis. The associated epibacterial population was largely dominated by bacteria which belong to the phylum *Firmicutes* (74%). Other phyla identified include *Proteobacteria* (18%), *Bacteroidetes* (4%) and *Actinobacteria* (4%). Up to seven different genera including *Micrococcus*, *Staphylococcus*, *Pseudorhodobacter*, *Winogradskyella*, *Alteromonas*, *Hoeflea* and *Paenibacillus* were found in the microbial community associated with intact *Laminaria digitata* samples (Figure 3.3).

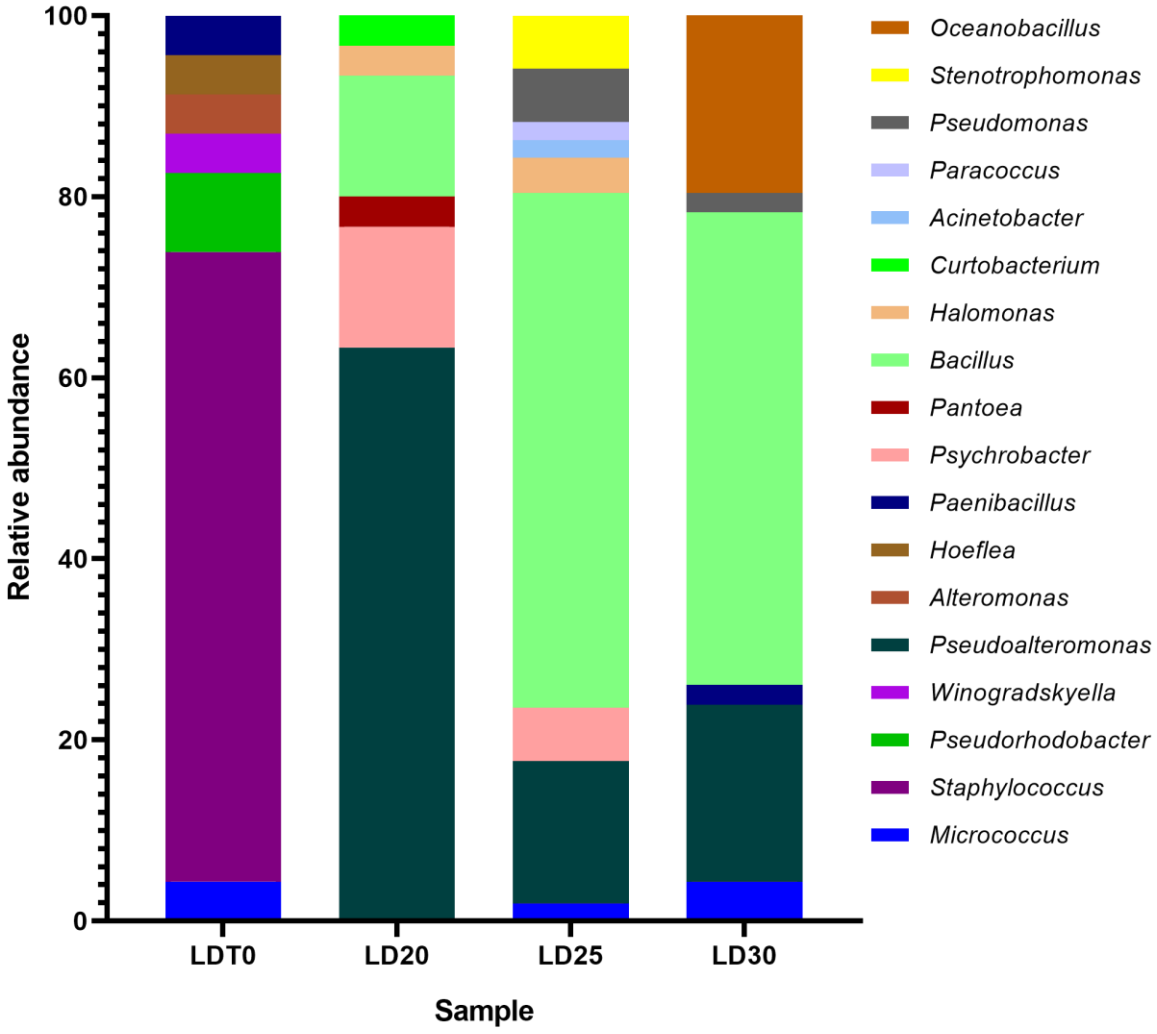

Figure 3.3: Relative abundances of bacterial genera associated with the cultivable surface microbiota of intact (LDT0) and *Laminaria digitata* decaying at 20 ˚C (LD20), 25 ˚C (LD25) and 30 ˚C (LD30). 16S rRNA gene sequences were obtained from the bacterial isolates and taxonomic analyses were performed. The relative distribution of phyla in each group is represented as a percentage.

3.4.7 Cultivable Surface Microbiota of decaying L. digitata

The ichip device loaded with cell-agar suspension from decaying seaweed samples collected at the three different temperatures was used to analyse the epibacterial population associated with decaying *Laminaria digitata.* This method was employed to potentially recover previously uncultivable bacteria which may have improved biological activities from the

environmental samples. The device was inoculated into each of the incubating flasks at the three different temperatures at week 2 and removed at week 4. Table 3.2 shows the number of bacterial isolates cultured from the three different incubating temperatures

Table 3.2. Number of bacterial isolates cultured from intact and decaying *Laminaria digitata samples*, incubated at different temperatures, using the maceration isolation method and the ichip device.

Intact seaweed (LDT0)	**Decaying seaweed**		
	20 °C	**25 °C**	**30 °C**
23	30	51	46

The cultivable surface microbiota of *L. digitata* decaying at 20 °C was found to consist of bacteria which belong to the phyla *Proteobacteria* (83%), *Firmicutes* (13%) and *Actinobacteria* (3%). Members of the genera *Pseudoalteromonas*, *Bacillus*, *Psychrobacter*, *Pantoea*, *Halomonas* and *Curtobacterium* were identified, with *Pseudoalteromonas* being the most abundant genus (Figure 3.3b). Similar bacterial phyla were also observed in the epibacterial population cultured from the macroalga decaying at 25 °C and 30 °C, with the exception of *Firmicutes* which was not present at 30 °C (Figure 3.3b and Figure 3.3c). The 25 °C derived microbial population was largely dominated by *Bacillus* species (57%) and also consisted of bacterial groups such as *Stenotrophomonas*, *Paracoccus*, and *Acinetobacter* which were not identified at other temperatures. The genus *Bacillus* also represented the most abundant group (52%) isolated from the decaying seaweed at 30 °C (LD30), together with other genera including *Oceanobacillus*, *Pseudoalteromonas*, *Micrococcus* and *Pseuodomonas* which were also present. The phylogenetic tree representing the bacteria cultured from both the intact and decaying seaweed at all three temperatures is shown in Figure 3.4.

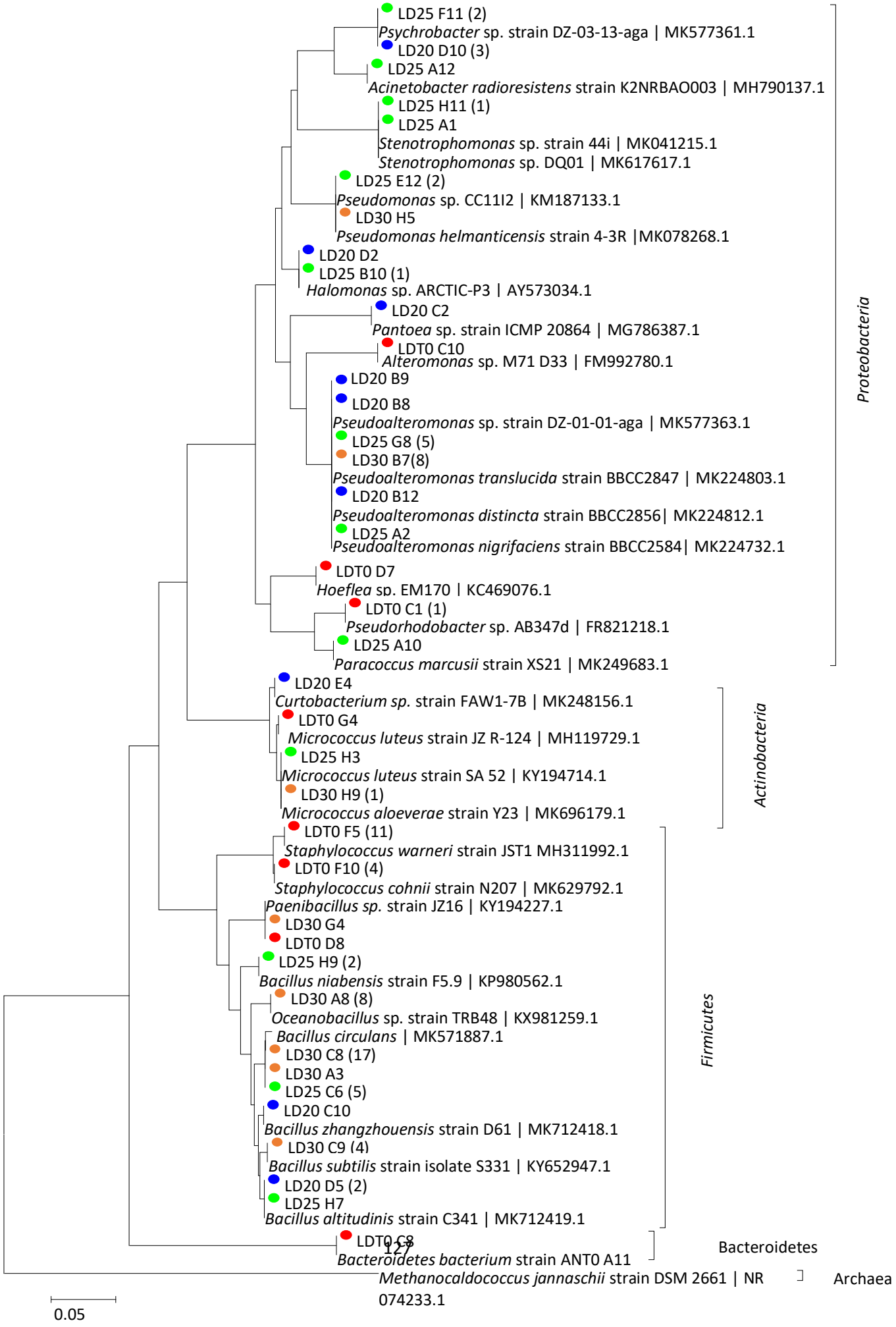
LD25 F11 (2)
Psychrobacter sp. strain DZ-03-13-aga | MK577361.1
LD20 D10 (3)
LD25 A12
Acinetobacter radioresistens strain K2NRBAO003 | MH790137.1
LD25 H11 (1)
LD25 A1
Stenotrophomonas sp. strain 44i | MK041215.1
Stenotrophomonas sp. DQ01 | MK617617.1
LD25 E12 (2)
Pseudomonas sp. CC11I2 | KM187133.1
LD30 H5
Pseudomonas helmanticensis strain 4-3R |MK078268.1
LD20 D2
LD25 B10 (1)
Halomonas sp. ARCTIC-P3 | AY573034.1
LD20 C2
Pantoea sp. strain ICMP 20864 | MG786387.1
LDT0 C10
Alteromonas sp. M71 D33 | FM992780.1
LD20 B9
LD20 B8
Pseudoalteromonas sp. strain DZ-01-01-aga | MK577363.1
LD25 G8 (5)
LD30 B7(8)
Pseudoalteromonas translucida strain BBCC2847 | MK224803.1
LD20 B12
Pseudoalteromonas distincta strain BBCC2856| MK224812.1
LD25 A2
Pseudoalteromonas nigrifaciens strain BBCC2584| MK224732.1
LDT0 D7
Hoeflea sp. EM170 | KC469076.1
LDT0 C1 (1)
Pseudorhodobacter sp. AB347d | FR821218.1
LD25 A10
Paracoccus marcusii strain XS21 | MK249683.1
LD20 E4
Curtobacterium sp. strain FAW1-7B | MK248156.1
LDT0 G4
Micrococcus luteus strain JZ R-124 | MH119729.1
LD25 H3
Micrococcus luteus strain SA 52 | KY194714.1
LD30 H9 (1)
Micrococcus aloeverae strain Y23 | MK696179.1
LDT0 F5 (11)
Staphylococcus warneri strain JST1 MH311992.1
LDT0 F10 (4)
Staphylococcus cohnii strain N207 | MK629792.1
Paenibacillus sp. strain JZ16 | KY194227.1
LD30 G4
LDT0 D8
LD25 H9 (2)
Bacillus niabensis strain F5.9 | KP980562.1
LD30 A8 (8)
Oceanobacillus sp. strain TRB48 | KX981259.1
Bacillus circulans | MK571887.1
LD30 C8 (17)
LD30 A3
LD25 C6 (5)
LD20 C10
Bacillus zhangzhouensis strain D61 | MK712418.1
LD30 C9 (4)
Bacillus subtilis strain isolate S331 | KY652947.1
LD20 D5 (2)
LD25 H7
Bacillus altitudinis strain C341 | MK712419.1
LDT0 C8
Bacteroidetes bacterium strain ANT0 A11
Methanocaldococcus jannaschii strain DSM 2661 | NR 074233.1
0.05
Proteobacteria
Actinobacteria
Firmicutes
Bacteroidetes
Archaea

Figure 3.4: Neighbor-joining phylogenetic tree representing bacterial phyla cultured from intact *Laminaria digitata* sample (red) and *Laminaria digitata* allowed to decay at 20 ^{o}C (blue), 25 ^{o}C (green) and 30 ^{o}C (orange) for four weeks. The evolutionary relationships of each phylum identified are shown with reference sequences from NCBI included. This phylogenetic analysis was made using single representative 16S rDNA sequences from each group identified by Avalanche NextGen Workbench version 2.30. This tree was drawn using MEGA program (version 7) and the number of similar sequences represented by each sequence is shown in brackets.

3.4.8 MAPD activities of L. digitata *Cultivable Surface Microbiota*

The cultivable surface microbiota isolated from both intact and decaying *Laminaria digitata* using the maceration method and ichip device respectively were screened for the production of macroalgal polysaccharide degrading enzymes, including lichenase, pectinase and hydroxyethyl cellulase activities. A combined total of 150 bacterial isolates (Table 3.2) were assayed using LB gellan gum plates containing the appropriate substrate. The bacterial population cultured from the seaweed prior to decay was found to consist of one bacterial strain identified as a *Paenibacillus* species which produced the three different enzymes screened for in this study under the conditions tested. Decaying *L. digitata* on the other hand was observed to be enriched in MAPD enzymes, with over 50% of the associated epibacterial community producing at least one of the enzymes screened in this study. From the 20 °C derived bacterial population, up to 83% produced lichenin degrading enzymes, while 23% and 57% were HE-cellulolytic and pectin lytic producing bacteria respectively. Over 40% of bacterial isolates cultured from the seaweed allowed to decay at 25 °C tested positive for lichenase, together with pectinase and HE-cellulose producing bacteria which were identified as 8% and 20% of

the microbial community respectively. The 30 °C derived population was equally active in algal cell wall degrading enzyme production, with more than 40% of the population being positive for at least one of the enzymes being tested. Overall, the major producers of these MAPD enzymes identified under the conditions tested in this study were *Pseudoalteromonas* and *Bacillus* species. In particular, two strains (LD25_B3 and LD20_C10) with efficient pectinase and lichenase activities were identified, and are being further characterized. The list of bacterial isolates cultured from both intact and decaying *Laminaria digitata* which produce at least one of the hydrolytic enzymes screened for are provided in Table S3.1 (Supplementary information).

3.5 Discussion

Seaweeds are well known to harbour diverse bacterial communities (Hollants et al., 2013), some of which can be beneficial or detrimental to their development (Goecke et al., 2010; Saha et al., 2011). Algal-associated bacterial populations have previously been studied using cloning, hybridization and various fingerprinting methods (Lachnit et al., 2009; Tujula et al., 2010). However, data on the detailed structure of the microbial communities found on weakened or decaying algae under different environmental conditions are still scarce. This work describes how *Laminaria digitata* associated epibacterial populations respond to changes that result from algal decay at different temperatures and helps to contribute to our current understanding of algal-bacterial interactions. The microbial communities of intact and decaying *Laminaria digitata* were studied using both metagenomic Illumina MiSeq sequencing strategies and culture dependent approaches. Bacteria belonging to six different phyla, including *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*, were identified. These group of bacteria have previously been identified amongst other *Laminaria* species (Staufenberger et al., 2008) and similar brown seaweeds including *Ascophyllum nodosum* (Ihua et al., 2019; Martin et al., 2015).

It is noteworthy that the bacterial community profiles observed in this study may have been affected by the culture conditions, including the isolation media and growth temperature (SYP-SW agar, 28 ^{o}C). In particular, certain phyla, such as *Planctomycetes* and *Chloroflexi*, identified in the metagenomic communities were not recovered in the cultivable epibacterial population, as these phyla would require several target isolation strategies to be captured (Jeske et al., 2015; Lage and Bondoso, 2012). Nonetheless, the results within the scope of methods employed demonstrate substantial changes within the epibacterial populations during decay and at different temperatures. The ichip device which has previously been used to isolate potentially novel bacteria from diverse environmental samples, some of which are applicable in enzyme-assisted extraction strategies (Ihua et al., 2019) was also applied in this study. While the device did not capture bacteria which may have been previously uncultivable, we isolated bacteria which displayed efficient algal cell wall polysaccharide degrading activities using the device. Some of these bacterial strains are being further characterized to test their EAE potential, among other biotechnological applications.

Exudates released during algal decay and the physiochemical changes which occur within the alga can influence the surrounding environment. Decaying *Cladophora* for example has been shown to result in acidic and anaerobic microcosm environments, together with increased nitrogen levels (Chun et al., 2017). In this study, the four week *Laminaria digitata* decay process has been shown to result in dynamic changes in the structure and composition of the bacterial communities associated with the brown seaweed. However, the results should be interpreted with caution as the experiments were not performed in replicates. The sequence data suggests that intact *Laminaria digitata* harbour a bacterial population which comprises of six different phyla, whereas only three phyla were observed during decay (Figure 3.1). However, it is important to note that the bacterial diversity measures calculated (Table 3.1) indicate greater species richness and diversity levels during decay but up to 20% of the

sequence dataset from decaying *L. digitata* remained unclassified. These unclassified sequences could potentially represent a diverse and complex group of bacteria yet to be included in the SILVA database (version 123) (Max Plank Institute, Bremen, Germany) (Quast et al., 2012) employed in this study. Nonetheless, these observed divergences in the bacterial taxonomic profiles found between the intact seaweed and its decaying counterpart can be attributed to environmental changes within the microcosm in the incubation flasks as a consequence of the algal decay which may favour the growth of certain groups of bacteria, as well as act as a selective pressure against some others. Previous studies have observed distinct differences in the algal associated microbial communities in algal species under healthy or weakened conditions. The epibacterial populations found on decaying *Ascophyllum nodosum*, for example, were found to be less abundant in *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes* and *Cyanobacteria* while a bloom in the presence of *Spirochaetae* and *Synergistetes* was observed, relative to the bacterial composition of intact *A. nodosum* in the same study (Ihua et al., 2019).

Pseudoalteromonadaceae and *Bacillaceae* represented the most abundant families identified in the bacterial communities associated with the brown seaweed in both the metagenomic communities and the cultivable surface microbiota population. In particular, the prevalence of these groups of bacteria was observed to become increasingly abundant during the decay period. Previous studies have reported the presence of *Pseudoalteromonas* and *Bacillus* species on the surface of seaweeds and other marine environments (Ihua et al., 2019; Ivanova et al., 1998; Kanagasabhapathy et al., 2009) and several authors have suggested that these bacteria employ efficient defence and competitive mechanisms which create a selective advantage for their proliferation under different environments. *Pseudoalteromonas* has been shown to produce a number of bioactive compounds which favour colonization of host surfaces and compete for available nutrients (Egan et al., 2001; Holmström and Kjelleberg, 1999) . *Bacillus*

species on the other hand can antagonize quorum sensing systems of other bacterial competitors by secreting a lactonase enzyme which degrades AHL involved in the detection and response of signalling molecules (Dong et al., 2002; Lee et al., 2002). In addition, these bacteria were also identified as the majority producers (accounting for up to 80%) of the algal cell wall degrading enzymes screened in this study. It is plausible that such effective hydrolysis of the carbohydrate rich algal cell wall contributed to the observed dominance of these bacterial species under the very nutrient limiting conditions employed in our experimental design.

The incubation temperature was another factor which appeared to contribute to differences in the microbial communities amongst decaying *Laminaria* samples. Certain temperature-related trends in the composition and relative abundance of the microbial consortia from the decaying seaweed were observed, with for example an increased prevalence of *Bacillaceae* at 30 ^{o}C and *Psychromonadaceae* at 20 ^{o}C (Figure 3.2). Environmental factors such as temperature, salinity and oxygen levels are well known to have an impact on the microbial communities associated with marine ecosystems (Lachnit et al., 2013; Schauer et al., 2003; Tujula et al., 2010). The impact of temperature on oxygen concentration, as described by Henry's law (Li et al., 2015) creates a selective pressure which is subsequently reflected in microbial communities that inhabit environments with different temperatures. Considering the ecological functions and contributions of these bacterial groups, changes in the epibacterial community due to temperature may result in shifts in the performance of the host alga.

Seaweeds have over many years developed a well-established symbiotic relationship with bacteria and other microorganisms. Seaweed associated bacterial communities can contribute to algal defence by producing antimicrobial and antifouling compounds (Dobretsov and Qian, 2002; Egan et al., 2013; Wilson et al., 2011). Algal associated epibacterial also produce plant growth-promoting substances, bioactive compounds, signalling and other effective molecules which are responsible for normal morphology, growth and development of their hosts (Singh

and Reddy, 2014). However, the marine environment consists of numerous bacterial species, some of which can be harmful to macroalgae. In order to defend against such harmful colonizers, macroalgae have thus developed several defence mechanisms, either through the production of antimicrobial compounds or induced defence through by leveraging certain beneficial bacterial relationships (Weinberger, 2007). The red alga *Bonnemaisonia hamifera* produces a potent metabolite (1,1,3,3-tetrabromo-2-heptanone) which decreases the number of bacterial epibionts and reduces its risk of bleaching (Nylund et al., 2008). Seaweeds also utilize metabolites which disrupt bacterial communication networks, such as quorum sensing systems that are responsible for bacterial biofilm formation and virulence. Hypobromous acid from *Laminaria digitata* has been shown to deactivate acylated homoserine-lactone (AHL) signals within the QS system (Borchardt et al., 2001) as a defence mechanism. From an ecological conservation perspective, it can thus be expected that only a minority fraction of algal associated epibionts will be capable of penetrating the cell wall and potentially cause damages to the algal host. This model was substantiated in this study, with less than 5% of the bacterial population isolated from the intact seaweed producing the algal cell wall degrading enzymes for which we screened. Consequently, under decaying conditions wherein the macroalgae and its defence systems are possibly weakened, the proportion of these MAPD bacteria can be expected to bloom, as was also observed in our study, contributing to algal biomass recycling (Martin et al., 2015). The presence of such algal cell wall degrading bacteria provides an entrance for opportunistic and pathogenic bacteria, thus being responsible for algal diseases (Egan et al., 2013; Goecke et al., 2010; Hollants et al., 2013). Therefore, it is not surprising perhaps that *Pseudoalteromonas* and *Bacillus* species which were identified as the most abundant and most represented MAPD bacteria during the decay period were scarcely present in the intact seaweed. Previous studies have also shown bacterial communities associated with healthy and weakened macroalgae to be functionally different (Chun et al., 2017; Marzinelli et

al., 2015), with more MAPD bacteria being isolated from *Ascophyllum nodosum* for example, during the algal decay than in the intact state of the seaweed (Ihua et al., 2019). These MAPD bacteria are of special interest as they represent a source of potentially novel enzymes applicable in a number of biotechnological industries, including enzyme assisted extraction strategies. MAPD bacteria isolated from decaying *Ascophyllum nodosum* have in fact been successfully shown to improve the extraction of useful phenolics from the brown alga *Fucus vesiculosus* with up to 65% extraction yields (Ihua et al., 2019).

In conclusion, the commercially relevant brown seaweed, *Laminaria digitata*, comprises of diverse and complex epibacterial communities which respond to both physiological and environmental changes, with respect to algal decay and temperature. These bacterial populations found in the decaying seaweed are also enriched with macroalgal polysaccharide degrading enzymes.

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Chapter 4: Morphological niches and seasonal variations drive the structure and composition of *Laminaria digitata* associated bacterial communities.

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4.1 Abstract

Stipitate kelp species such as *Laminaria digitata* dominate most subtidal rocky shores and are among the most productive members of kelp forests worldwide. *Laminaria* also sustains rich bacterial communities which offer a variety of biotechnological applications. However, to date, in-depth studies on the diversity and uniqueness of bacterial communities associated with these macroalgal species, their ecological role and their interactions with the algae are under-represented. To address this, the diversity of bacterial populations associated with different morphological parts (holdfast, stipe, meristem, blades) of this brown seaweed was investigated using high-throughput Illumina sequencing of the 16S rRNA genes. The epibacterial communities of the brown seaweed were found to be specific, with the core community comprising of less than 3.5 % of the total amplicon sequence variants. The diverse holdfast and blade tissues formed distinct clusters while the meristem and stipes regions were found to be more closely related. We also examined the variations in the microbial community structure of each thallus part over a ten month period, differential prevalence of bacterial groups across seasons was evident, with the microbiome of algal tissues obtained in November being identified as the most diverse when compared to other months. The data obtained further supports the hypothesis that macroalgal bacterial communities are shaped by morphological niches and display specificity.

4.2 Introduction

Seaweeds represent a unique source of high value hydrocolloids, such as agar, carrageenan and alginate, which have a number of biotechnological applications (Armisen and Gaiatas, 2009; Buck et al., 2006; Szekalska et al., 2016). The global alginate market alone was valued at USD624.0 million in 2016 and it is estimated that it will be worth USD923.8 million by 2025 (Research, 2017a; Research, 2017b). Overall, hydrocolloids obtained from seaweeds have a commercial value of up to US$1 billion which is also projected to further increase (Bixler and Porse, 2011). Extracts from seaweeds have been reported to possess various pharmaceutical properties such as antiviral (Wang et al., 2012), anti-inflammatory (Zhang et al., 2014) and anticoagulant activities (Adrien et al., 2017), together with being demonstrated to alleviate abiotic stress in plants (Goñi et al., 2016). Laminarin derived from *Laminaria digitata* has for example, been demonstrated to reduce the infection of *Plasmopara viticola*, a disease-causing fungus; on grapevine by 75% by eliciting several defence mechanisms such as optimizing calcium influx, alkalinization of the extracellular medium and activation of defence-related genes (Aziz et al., 2003). Marine macroalgae have thus gained increased attention in recent times. This is evidenced by an increase in the harvesting and cultivation of seaweeds of over 9 million tonnes between 2001 and 2010, resulting in an increase in revenue of more than US $ 2,375M (Ghadiryanfar et al., 2016). This increased seaweed farming has had the additional beneficial effect of lowering overfishing rates, as well as improving the socio-economic lifestyle of communities in coastal regions (Flower, 2011).

In addition, the high nutritional value of these marine species cannot be overemphasized. Brown seaweeds represent the most edible species consumed as a dietary component in Asia and are gaining popularity elsewhere as useful sources of macro and micronutrients, including ash (21.1-39.3%) and sulphate (1.3-5.9%) (Rupérez, 2002). From a nutritional standpoint, *Ascophyllum nodosum* has been reported to have an overall protein, lipid and ash content of

8.7 g/100 g DW, 3.6 g/100 g DW and 30.8 g/100 g DW respectively. The total amino acid content of *A. nodosum*, 7.48 g/100 g DW, indicates a negligible level of non-protein nitrogenous materials in the alga. The brown alga *Fucus vesiculosus* also contains good levels of minerals, with levels of Ca (1160.3mg/100g DW), Fe (19.0mg/100g DW), K (3745.0mg/ DW) and Na (2187.5mg/100g DW) being reported (Lorenzo et al., 2017). Seaweeds are also desirable marine species for use as feedstock for ethanol fermentation as well as anaerobic digestion for methane production due to their rich carbohydrate content (Kraan, 2013). *Laminaria digitata* which contains up to 57% glucose, has in particular been identified as one of the most promising brown seaweed biomass sources for biomethane production (Hou et al., 2015). The lack of lignocellulose and the possibility of enzymatic saccharification at lower temperatures have also positioned marine macroalgae as a potentially better and more environmentally friendly alternative to the use of land plants for ethanol production (Sharma and Horn, 2016).

Marine ecosystems consist of a rich repertoire of microbial life, with over one million different marine species present (Whitman et al., 1998). Seaweeds play a major role in the marine environment as global primary producers which are responsible for the cycling and maintenance of nutrients in the ocean. Owing to the carbon rich nature of the algal cell walls and the physicochemical properties of seaweed surfaces, they also provide a suitable substratum for colonization by epibionts as well as being a shelter for a number of invertebrate species (Burke et al., 2011b; Goecke et al., 2010; Wiese et al., 2009). Seaweed-bacterial relationships can be beneficial in nature, with bacteria demonstrating positive effects relating to host health, development and defence (Egan et al., 2013; Goecke et al., 2010). Metabolites such as thallusin from epiphytic marine bacteria have for example been shown to induce differentiation and germination in *Monostroma oxyspermum* and other green macroalgae (Matsuo et al., 2005). *Pseudoalteromonas* species which are frequently isolated from algal

surface have also been shown to possess antifouling properties which protect their algal hosts from invertebrate larvae, benthic diatoms and different bacterial settlement (Bowman, 2007, Egan et al., 2001). Furthermore, *Pseudoalteromonas porphyra* isolated from *Laminaria japonica* has been reported to promote growth and spore germination in the alga (Dimitrieva et al., 2006).

Variations in macroalgal epibacterial communities have previously been observed within different parts of host algal species. These variations are known to be influenced by environmental factors such as light, temperature, salinity and dissolved nutrients, biogeography; host phylogeny and functional differences between morphological distinct parts of the algae (Morrissey et al., 2019). For example, microbial communities found on the meristem and cauloid parts of *Saccharina lassitima* were found to be more closely related, regardless of geographical region or seasonal influences; whereas the epibacterial populations found on the phyloid and rhizoid were considerably different (Staufenberger et al., 2008). Variations in microbial community structure in the canopy forming kelps *Nereocystis luetkeana* and *Macrocystis pyrifera* have been reported to be dependent on the age of the blade tissue, geographical location and host species identity (Weigel and Pfister, 2019). While host species, biogeography together with nutrient levels have also been shown to contribute to the bacterial communities in the green seaweeds *Caulerpa prolifera* and *Caulerpa cylindraecea* (Morrissey et al., 2019).

Previous studies on *L. digitata* reported that while the bacterial communities associated with the blade region of this brown alga were found to be diverse, they were functionally different from the microbial communities present on the metabolically inactive aged peripheral tissues of the macroalga (Salaün et al., 2010; Salaün et al., 2012). This variation in seaweed microbial populations between different morphological parts of macroalgae may be due at least in part to the patterns of vascular architecture in seaweeds. Honkanen and Jormalainen (2005) suggests

that different parts of most seaweeds exhibit independence in the absorption of nutrients and the production of photosynthates, thus lack vascular connections for efficient resource translocation. While the distribution and abundance of marine macroalgal associated epibacterial communities is as previously mentioned driven by different factors, seasonal variations have in particular been found to play a significant role in the structure of algal associated microbial communities (Burke et al., 2011b; Staufenberger et al., 2008).

Seasonal variations have also been reported to impact the biochemical composition of seaweeds (Schiener et al., 2015; Tabassum et al., 2016). Chemical profiling of *Laminaria* species revealed an accumulation of laminarin and mannitol during summer and autumn months, whereas ash levels were found to be highest during colder seasons (Adams et al., 2011; Schiener et al., 2015). The total dietary fibre content of *Sargassum horneri* has been shown to reach a maximum in spring (Murakami et al., 2011), while the levels of growth-stimulating polyamines in *Ecklonia maxima* have also been reported to correlate with seasonal variations in seawater temperature (Papenfus et al., 2012). While there are ongoing studies on the seasonal variation of macroalgal chemical composition, there are still knowledge gaps in the direct correlation of algal biochemistry to its associated bacterial population. It is, as of yet, unknown whether or not microbial community structures, or the presence or absence of particular microbial taxa at different seasons can be used as a proxy indicator of specific biochemical properties of seaweeds. Therefore, describing *L. digitata*-associated community to identify bacterial groups correlated to seasonality of sampling may be a step in linking algal microbiota to physiology and aid the prediction of ideal seaweed harvesting times for accessing nutraceuticals or pharmaceuticals.

In this study, we report on the diversity and composition of the microbial communities of the brown alga *Laminaria digitata* sampled at different periods over a 10 month period (April 2016, July 2016, November 2016 and January 2017) investigated using high throughput

Illumina MiSeq sequencing. Significant differences in the bacterial community structure associated with the algal holdfast, stipe, meristem and blade regions were observed. Taxonomic analysis demonstrates that bacterial populations associated with *L. digitata* displayed significant morphological variation and were differentially abundant over different seasons, with the holdfast region in *L. digitata* being identified as the most diverse tissue, whereas the meristem region was the least diverse. Overall, we report that *L. digitata* associated bacterial populations demonstrate high specificity to thallus part and season as there appeared to be a lack of a core bacterial community – only less than 3.5 % of amplicon sequence variants were common to all the algal samples.

4.3 Materials and Methods

4.3.1 Sampling

Samples of *Laminaria digitata* were collected from Finavarra in Co. Clare, Ireland at 53° 08' 59'' North, 9° 08' 09'' West in April 2016, July 2016, November 2016 and January 2017. The different sections (blade, stipe, meristem and holdfast) were sampled from a mixture of 4 to 8 different algal thalli at each sampling time to ensure a good representation, rinsed in sterile water to remove loosely attached particles and transferred into sterile air-tight plastic bags. The graphical representation of these different morphological parts of *Laminaria digitata* is shown in Figure 4.1 and the sample metadata is provided in Table S4.1 (Supplementary information). All samples were kept on dry ice at the sampling location and subsequently stored at either -20 °C or -80 °C, in the laboratory before further analyses.

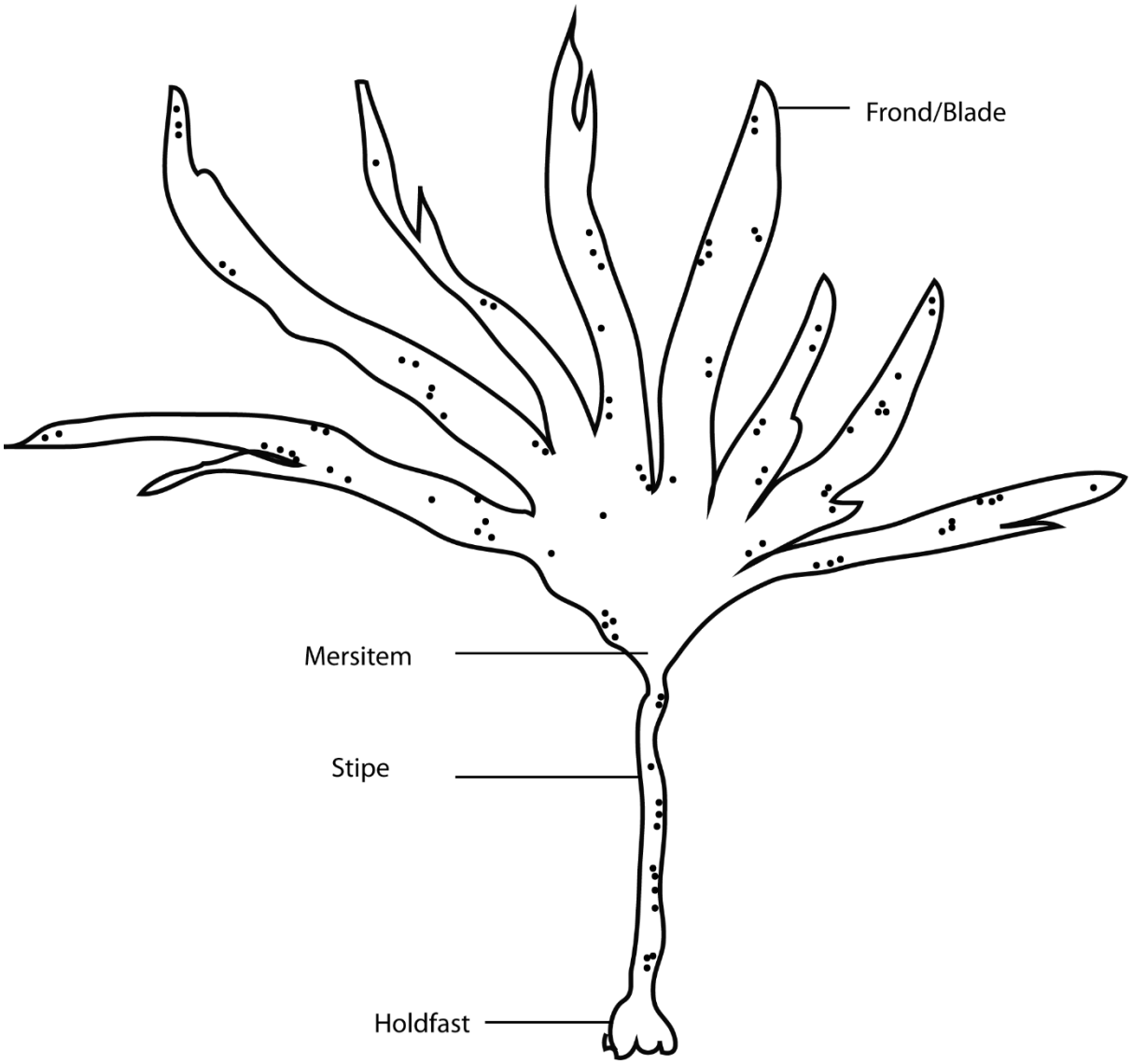

Figure 4.1: Four morphological parts of a *Laminaria digitata* thallus

4.3.2 DNA extraction

Metagenomic DNA was extracted from approximately 0.5 g of each *Laminaria* sample which was crushed into a fine powder under liquid nitrogen using a sterile mortar and pestle. The ground powder was suspended in 500 ml of CTAB lysis buffer (2 % *w/v* CTAB, 2 % *w/v* polyvinylpyrrolidone, 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris HCl pH 8.0), bead-beaten for 60 seconds with sterile beads and incubated at 65 °C for 60 minutes, with occasional mixing. Equal volume of phenol chloroform isoamyl alcohol was added and the mixture was

centrifuged for 30 minutes at 4,300 x *g*. The aqueous phase was collected into a separate eppendorf tube, and the DNA was precipitated with 0.7 volume of ice cold isopropanol and 0.1 volume of sodium acetate (3 M, pH 5.2) and centrifuged (at 20 ℃) for 30 minutes at 4,300 x *g*. The pellet was washed twice in 70% *v/v* ice cold ethanol, air dried and re-suspended in TE buffer. DNA was visualized using agarose gel electrophoresis and quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific, Delaware, USA). For each algal part (blade, stipe, meristem and holdfast), metagenomic DNA was extracted from three separate individual samples obtained at each time point. DNA from each of the three individuals obtained for each part, at each time point, were then subsequently pooled together to represent a working template for PCR amplification.

4.3.3 16S rRNA gene amplification and MiSeq Sequencing

PCR amplification of the V3-V4 16S rRNA gene region was performed using forward (5'-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*CCTACGGGNGGCWGCAG -3') and reverse (5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GACTACHVGGGTATCTAATCC -3') primers (Klindworth et al. 2013); which contained Illumina adapter overhang sequences (written in italic text) as previously described (Ihua et al., 2019). This set of primers have previously been identified as being efficient in representing good levels of bacterial diversity and have been used in a wide range of environmental studies (Klindworth et al., 2013, Logue et al., 2012, Abrahamsson et al., 2012). PCR amplification was performed under the following conditions: 98 ˚C for 30 s, followed by 30 cycles of denaturation (98 ˚C for 10 s), primer annealing (57 ˚C for 30 s), primer extension (72 ˚C for 30 s), and 72 ˚C for 5 min. PCR amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter) according to the manufacturer's instructions and a subsequent reduced-cycle (8 cycles) reaction was performed to further attach unique dual eight-base Nextera XT multiplexing indexes and

sequencing adapters under similar cycling conditions. Index PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter; Fisher Scientific, Dublin, Ireland) according to the manufacturer's instructions. All PCR reactions for each *Laminaria* thallus part sampled at each time point were performed in triplicates, pooled together and sequenced using Illumina Miseq 300bp paired-end sequencing by Macrogen (Seoul, Korea).

4.3.4 Sequence data processing and Statistical analyses

Raw sequence data obtained were de-multiplexed according to their unique barcodes and processed using Trimmomatic (version 0.38) (Bolger et al., 2014) to remove end positions with a quality score below 25. The R package DADA2 (Callahan et al., 2016) was then used to exclude primer sequences, filter and de-noise sequences, de-replicate unique amplicon sequence variants (ASVs, similar to 100%-identity operational taxonomic units), and remove bimeric sequences. R package DADA2 default settings where employed for these analysis, with the exception of maximum error (maxEE=c(3,4)). Taxonomic identities of ASVs were assigned using the R package DECIPHER (Wright, 2016) and the SILVA 132 database release (Quast et al., 2012). Chloroplast sequences were identified were and removed from downstream analyses. Table 4.1 shows the number of raw and quality filtered sequences, together with final sequencing depth of each sample. Ecological metrics were calculated using the R package vegan (Oksanen et al., 2014), and read counts were normalised using the R package GMPR (Chen et al., 2018). Differential testing between conditions was carried out using ADONIS2 and ANOSIM (R package vegan) (Oksanen et al., 2014), as well as a Kruskal-Wallis test for differences between groups with post-hoc Dunn test (R package FSA) (Ogle et al., 2019). Cluster analysis of samples by Bray-Curtis distance was carried out via Ward-linkage (*hclust*, method = "ward.D") (Murtagh and Legendre, 2014), while abundant ASVs (at least 300 reads in at least 15% of samples) were clustered using Spearman correlation. A correlation network between features was constructed using Spearman's correlation coefficient

and the R package igraph (Csardi, 2019). Relative abundances were compiled and presented in Graphpad (Prism, 1994), while community features were visualised and explored using the R packages ggplot2, phyloseq, complexheatmap, vegan, gplots, venneuler and reshape2 (Gu et al., 2016, McMurdie and Holmes, 2013; Oksanen et al., 2014; Warnes et al., 2009; Wickham, 2016; Wickham, 2017; Wilkinson and Urbanek, 2011).

Table 4.1: Number of raw and quality filtered sequences, with final sequencing depth of bacterial communities associated with the holdfast (HF), stipes (SP), meristem (MST) and blades (BD) of *L. digitata* sampled in April (APR), July (JULY), November (NOV) and January (JAN)

Sample ID	**Number of raw sequences**	**Number of final sequences (filtered, chloroplast removed)**	**Final sequencing depth**
APR-16-HF	1,473,696	427943	428101
APR-16-SP	1,331,760	329528	329665
APR-16-MST	1,168,362	390870	390870
APR-16-BD	1,391,010	357365	357812
JULY-16-HF	1,530,674	424983	425281
JULY-16-SP	1,411,586	488396	488556
JULY-16-MST	1,192,636	345795	345889
JULY-16-BD	1,568,430	413029	414162
NOV-16-HF	1,199,686	339680	339904
NOV-16-SP	1,452,524	429593	429855
NOV-16-MST	1,418,620	494055	494110
NOV-16-BD	1,419,686	329335	329503
JAN-17-HF	1,315,864	420566	420810

JAN-17-SP	1,275,340	451058	451087
JAN-17-MST	1,398,882	498516	498528
JAN-17-BD	1,322,862	428145	428182

4.3.5 Accession number

The metagenomic sequencing data was deposited in the European Nucleotide Archive (ENA) under the accession number ERX3500545 - ERX3500549, ERX3500553-ERX3500560 and ERX3524768 - ERX3524771.

4.4 Results

4.4.1 Diversity and species richness of L. digitata *communities*

Epibacterial communities derived from different parts of *L. digitata*, collected at four different sampling times over a 10 month period, were analysed to determine if differences existed in their overall structure, diversity and composition. The diversity of the bacterial communities were examined using Shannon and Inverse Simpson indices represented in Figure 4.2. Overall, the *L. digitata* holdfast tissues displayed the highest level of species richness, closely followed by the algal blades, while samples obtained in November harboured the most diverse bacterial community. Meristem tissues were also found to be the least diverse, while stipes displayed medium levels of diversity (Figure 4.2). The stipe and blade tissue samples displayed a similar pattern, with a continuous rise in diversity from April to November and a subsequent decline in January. Maximum richness levels were observed in April for the holdfast tissue samples, but this declined between July and November and were seen to recover in January. The epibacterial populations isolated from the meristem on other hand was least diverse in April, with highest levels being recorded in July.

In addition, it is important to note that the bacterial diversity trends noted in the algal-derived bacterial communities do not match the patterns of the sequencing depth of the individual algal samples (Figure 4.2). Considering that the read counts were normalized prior to analysis, this

indicates that the levels of species richness that we observed is not a consequence of a greater number of sequences being available in any given sample and this increases our confidence in the interpretation of the results.

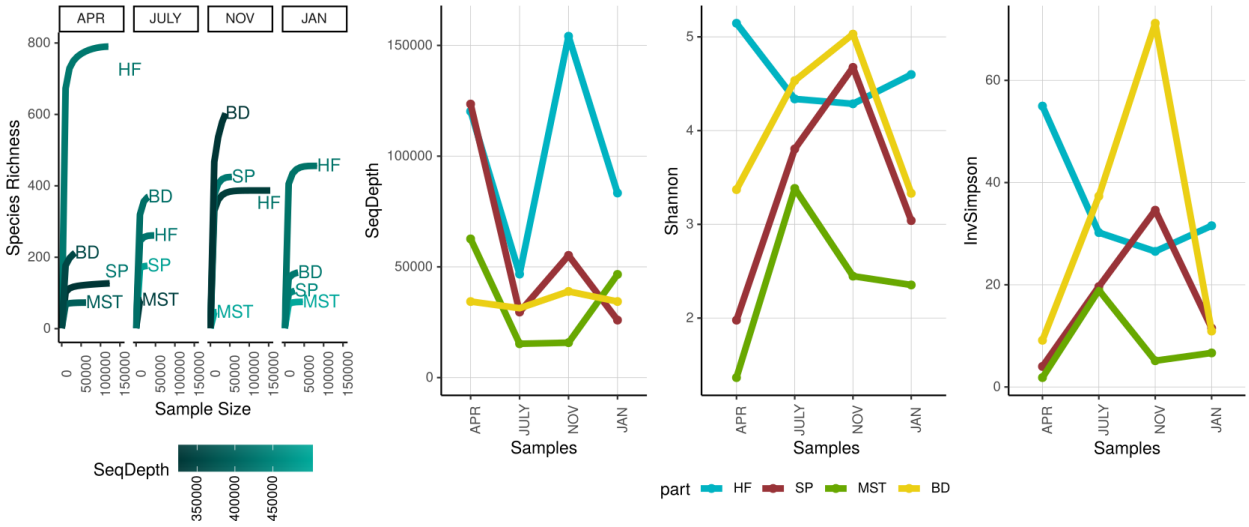

Figure 4.2: Sequencing depth, species richness and alpha diversity measures of the microbial communities associated with holdfast (HF), stipes (SP), meristem (MST) and blades (BD) of *L. digitata* collected in April (APR), July (JULY), November (NOV) and January (JAN).

4.4.2 Seasonal and spatial variations in L. digitata *associated microbial communities*

The bacterial communities associated with *L. digitata* were shown to consist of bacteria which belong to the phyla *Proteobacteria* (40 %), *Planctomycetes* (36 %), *Bacteroidetes* (12 %), *Actinobacteria* (8 %), *Verrucomicrobia* (2 %), *Cyanobacteria* (1 %) and *Firmicutes* (<1 %). Greater variation and bacterial diversity was observed at the lower taxonomic genus level, with up to 21 genera, including *Psychrobacter*, *Blastopirellula*, *Flavobacterium*, *Litorimonas* and *Hellea*, being identified (Figure 4). Up to 15 % of the bacterial ASVs remained unclassified at the genus level. These unclassified ASVs are likely to represent groups of bacteria currently unavailable in the SILVA database (version 132) (Quast et al., 2012) which we employed in this study, or possibly novel bacterial species.

Distinct differences were however observed between the epibacterial microbial populations associated with the four different parts of the brown seaweed and between the four different sampling times (Figure 4.3).

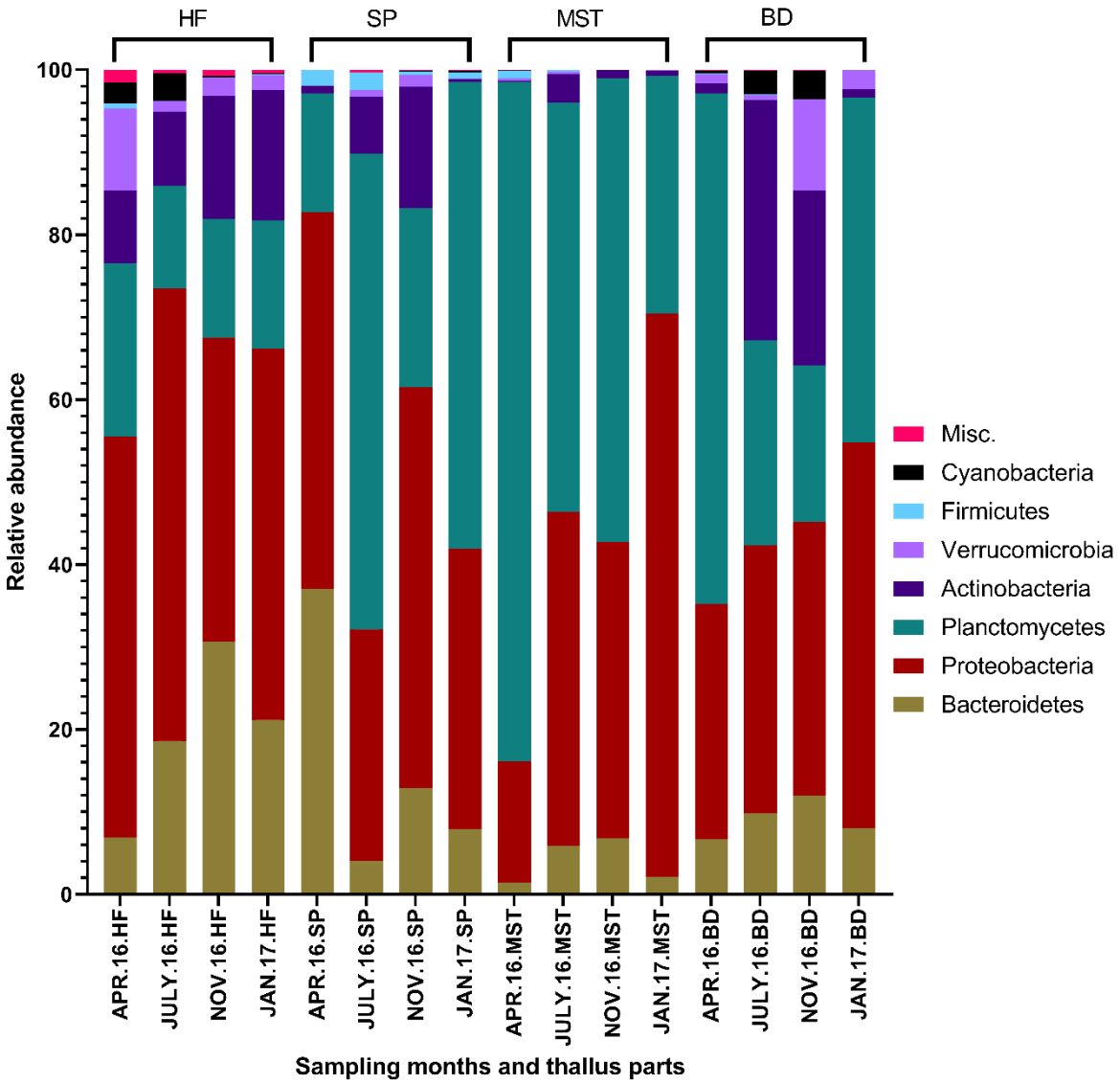

Figure 4.3: Relative abundance at phylum level of the holdfast (HF), stipes (SP), meristem (MST) and blades (BD) of *L. digitata* collected in April (APR), July (JULY), November (NOV) and January (JAN). Misc; Miscellaneous (bacterial phyla with less than 0.005 rank mean sample relative abundance).

4.4.2.1 Holdfast

The epibacterial communities associated with the holdfast tissue were diversely comprised of up to 12 different genera, the major ones being *Blastopirellula, Aquamarina,* SVA0996 marine group, *Roseobacter* and *Litoreibacter.* In particular, the majority of the bacterial sequences obtained from the holdfast samples were unclassified at the genus level or grouped as 'miscellaneous' which represents a combination of many different genera which are present in the metagenomic community but at less than 0.005 rank mean sample relative abundance (Figure 4.4). Members of the genus *Aquimarina* consistently increased in relative abundance between April 2016 and November 2016 but decreased thereafter in January 2017. Similarly, the prevalence of the SVA0996 marine group was also observed to increase throughout the sampling months. At the phylum level, *Proteobacteria* dominated the dataset, while *Firmicutes* and *Cyanobacteria* were the least observed.

4.4.2.2 Stipes

The bacterial populations associated with the stipe samples were found to comprise of members of the phyla *Proteobacteria* (39 %), *Planctomycetes* (38 %), *Bacteroidetes* (15 %), *Actinobacteria* (6 %) and *Firmicutes* (1 %). The data set in April was diversely populated, with the major genera present being *Winogradskyella* (36 %; phylum *Bacteroidetes*), *Celeribacter* (32 %), *Granulosicoccus* (3 %) and *Litorimonas* (3 %) which belong to the phylum *Proteobacteria,* together with *Blastopirellula* (14 %; phylum *Planctomycetes*). The prevalence of *Blastopirellula* and *Granulosicoccus* further increased in July, with *Rhodopirellula* also beginning to emerge. Members of the genera *Streptomyces* (1.7 %) and *Litoreibacter* (4 %) were found in the November samples, but the majority of the sequences identified in this month were unclassified at the genus level or grouped as miscellaneous. The unclassified sequences are likely to represent members of *Actinobacteria* (14 %) and *Verrucomicrobia* (1.5 %) which were identified in November at the phylum level (Figure 4.3). The epibacterial communities

associated with the stipe tissue samples from the final month (January 2017), were dominated by bacteria from the *Blastopirellula* genus.

4.4.2.3 Meristem

The prevalence of the phylum *Planctomycetes* was evident in April (82 %), July (50 %) and November (29 %) samples, while *Proteobacteria* (68 %) dominated the meristem-derived microbiota in January 2017. The *Blastopirellula*-prevalent bacterial population in April succeeded into a more diverse population in July, with members of the genera *Litorimonas, Granulosicoccus, Portibacter* and SVA0996 marine group becoming more evident. Furthermore, *Litorimonas* was even more abundant in January 2017, together with bacterial sequences identified as *Hellea* and *Granulosicoccus*.

4.4.2.4 Blades

While members of the phylum *Planctomycetes* (62 %), being mainly represented by the genus *Blastopirellula*, followed by *Proteobacteria* (29 %) dominated the microbial population in April, *Bacteriodetes* (7%) was also present but very low levels of *Verrucomicrobia* (1 %) and *Actinobacteria* (1 %) were observed. However, a decrease in the prevalence of *Planctomycetes* (25%) was noted in July as levels of *Actinobacteria* (29 %) increased, resulting in a more evenly structured bacterial community. At the genus level, the presence of *Litorimonas, Rhodopirellula* and SVA0996 marine group was also observed to be abundant in July. The composition of the bacterial community remained largely unchanged in the blade samples in subsequent months until January, but levels of *Litorimona*s and *Granulosioccus* were higher in January (13 % and 18 % respectively) than in November (6 % and 2 % respectively).

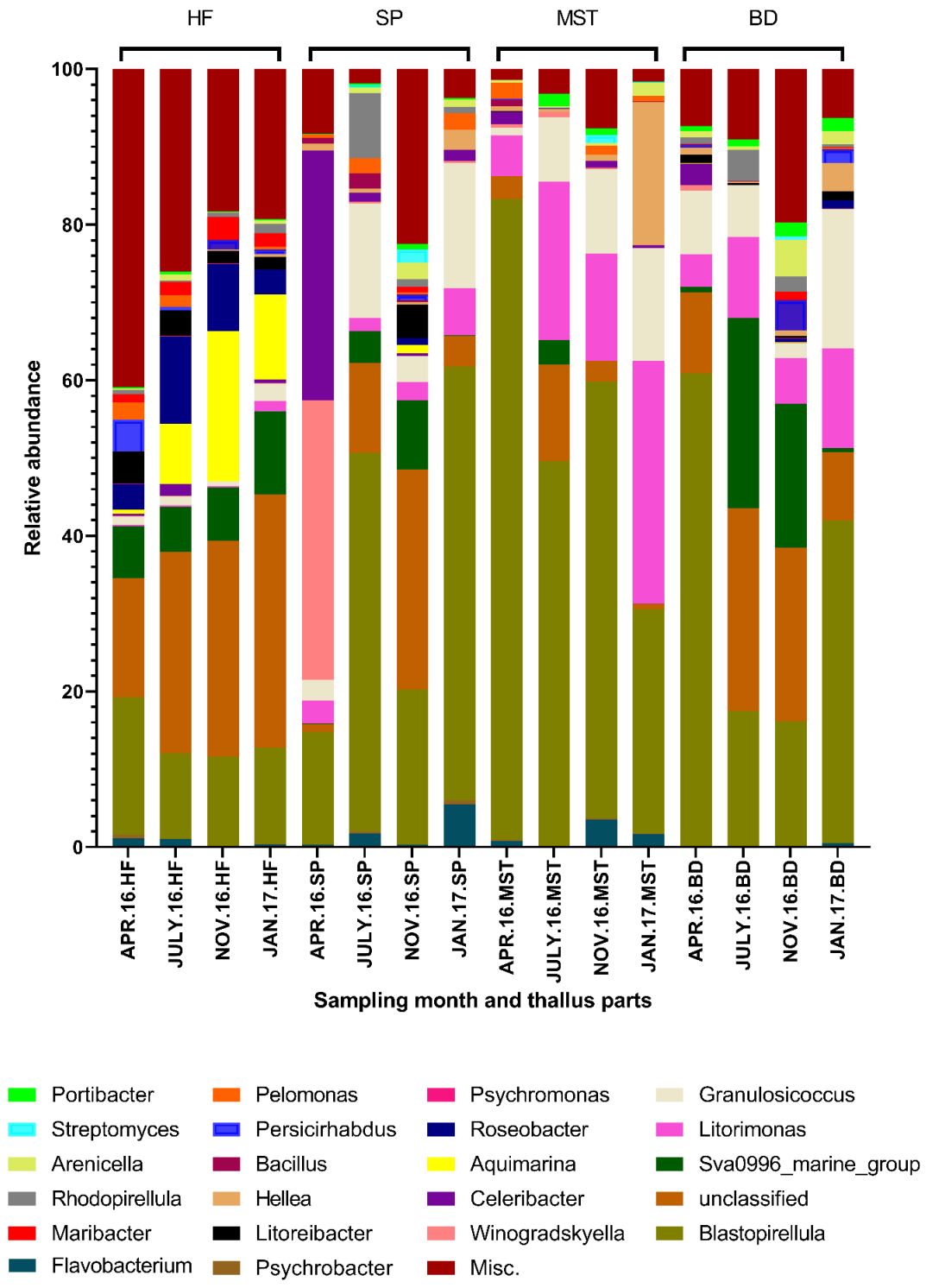

Figure 4.4: Relative abundance at genus level of the holdfast (HF), stipes (SP), meristem (MST) and blades (BD) of *L. digitata* collected in April (APR), July (JULY), November (NOV) and January (JAN). Misc; Miscellaneous (bacterial phyla with less than 0.005 rank mean sample relative abundance).

4.4.3 Comparative analysis of **L. digitata** *associated bacterial populations*

Differences in community composition (beta diversity of Bray-Curtis dissimilarity values) are represented in the PCoA ordination plot (Figure 4.5), with the first two axes accounting for 48% of the variation observed. The greater part of this variance (30%) is accounted for by the first axis and displays differentiation of the samples by their thallus parts whereas the second axis (18%) highlights the differences observed between the sampling months. The distance between the positions of any two plots represent the extent of their similarity in bacterial composition. A Kruskal-Wallis test (Supplementary information Table S4.2; chi-squared = 11.404, df = 3, p value = 0.009729) of the variance contained in these first two axes revealed statistically significant differences between the epibacterial communities associated with the four different algal thallus parts. Across all sampling months, the holdfast-related bacterial communities appear to associate strongly, forming a distinct cluster which is separate and distantly located from the other bacterial populations (Figure 4.5). Similarly, the bacterial communities of blades sampled at different months displayed strong similarities and formed a separate clusters. However, the blades-associated microbial population formed two distinct clusters based on their sampling month; April clustered with January, while July clustered with November. Interestingly, all stipe samples failed to cluster together and likewise meristem samples which were collected in April and July remained separate on the PCoA plot. However, meristem samples obtained in July and November appeared to group together. Notwithstanding, the microbial populations from meristem and stipes are seen to be more closely related than they are to holdfast and blades related communities. Furthermore, the Dunn test identified holdfast-stipes (Supplementary information Table S4.2; Z score = -2.451, adjusted p value = 0.043) and holdfast-meristem (Supplementary information Table S4.2 Z score = -3.193, adjusted p value = 0.008) as thallus pairs which account for the significant differences in associated epibacterial populations observed between the algal parts. The fact that holdfast is present in both significant pairs, while meristem-stipes (Supplementary

information Table S4.2 Z score = 0.743, adjusted p value = 0.458) is not significantly different indicates the holdfast is the most significantly different part. The Kruskal-Wallis test was also used to assess the statistical significance of the differences between the microbial populations of *L. digitata* obtained, taking account of the sampling months; as shown in the PCoA plot (Figure 4.5). While no statistically significant differences (Supplementary information Table S4.3; chi-squared = 1.8309, df = 3, p value = 0.6082) were observed, slight season-related trends were nonetheless observed.

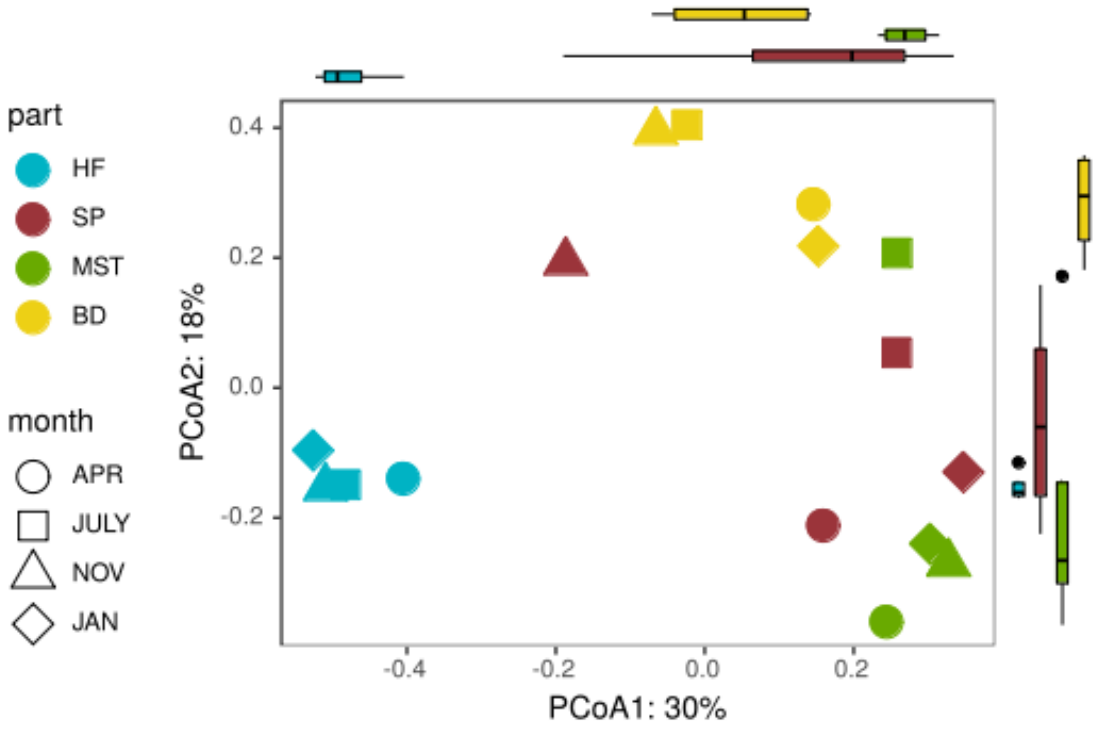

Figure 4.5: Principal coordinate analysis (PCoA) plot based on Bray-Curtis distance matrix of the epibacterial communities associated with the holdfast (HF), stipes (SP), meristem (MST) and blades (BD) of *L. digitata* collected in April (APR), July (JULY), November (NOV) and January (JAN). The margins show how well the sampling groups (particularly "part") separated within the first two axes, with a boxplot (middle is median, outer margins are 25th & 75th

percentiles; whiskers cover points within 1.5 interquartile ranges) emphasizing the segregation of samples based on seaweed part.

4.4.4 Core and unique epiphytic bacterial communities on L. digitata

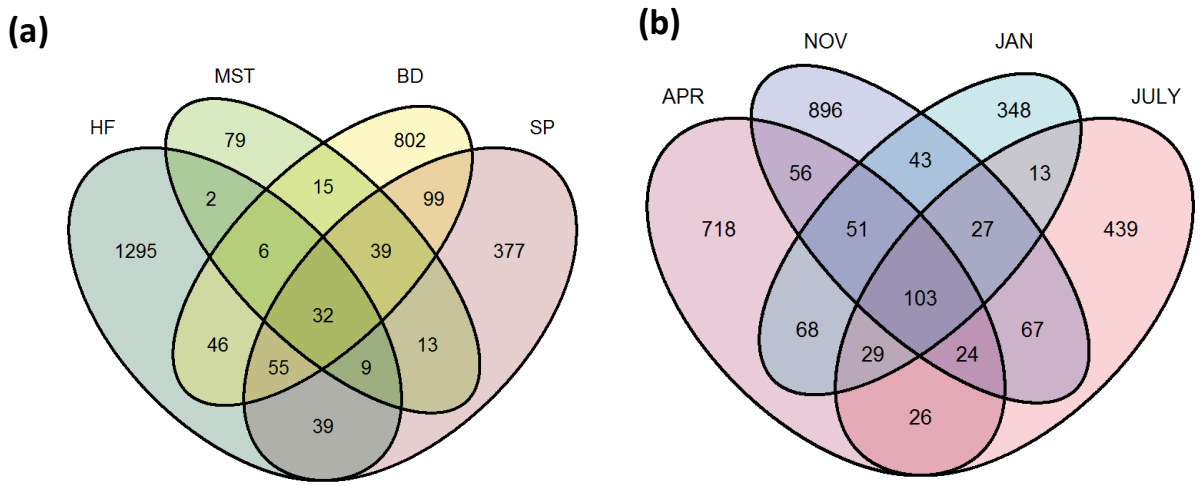

Figure 4.6: Venn diagram showing unique and overlapping amplicon sequence variants (ASVs) identified in *L. digitata* bacterial communities categorized by (a) parts and (b) sampling month. Abbreviations; holdfast (HF), meristem (MST), blades (BD) and stipes (SP), April (APR), July (JULY), November (NOV) and January (JAN).

The epibacterial communities which are unique to each algal thallus part (Figure 4.6a) and sampling month (Figure 4.6b), as well as bacterial species shared across all samples were analysed. The holdfast tissue displayed the highest number of unique ASVs (45 %; 1295 ASVs), suggesting a microcosm suitable for the colonization of a specific subset of bacterial species. Only 2.7 % (79 ASVs) on the other hand were shown to be unique to the meristem region. Blades and stipes were found to harbour 802 ASVs and 377 ASVs respectively. More ASVs were found to be specific to November (896 ASVs) than in other sampling months. Interestingly, *L. digitata* surface-attached bacterial populations were found to be specific to the algal part and season, with only 1.1% and 3.5% ASVs being shared between the algal samples

according to their parts and sampling months respectively. The majority of these shared communities were further identified as belonging to the genus *Blastopirellula*.

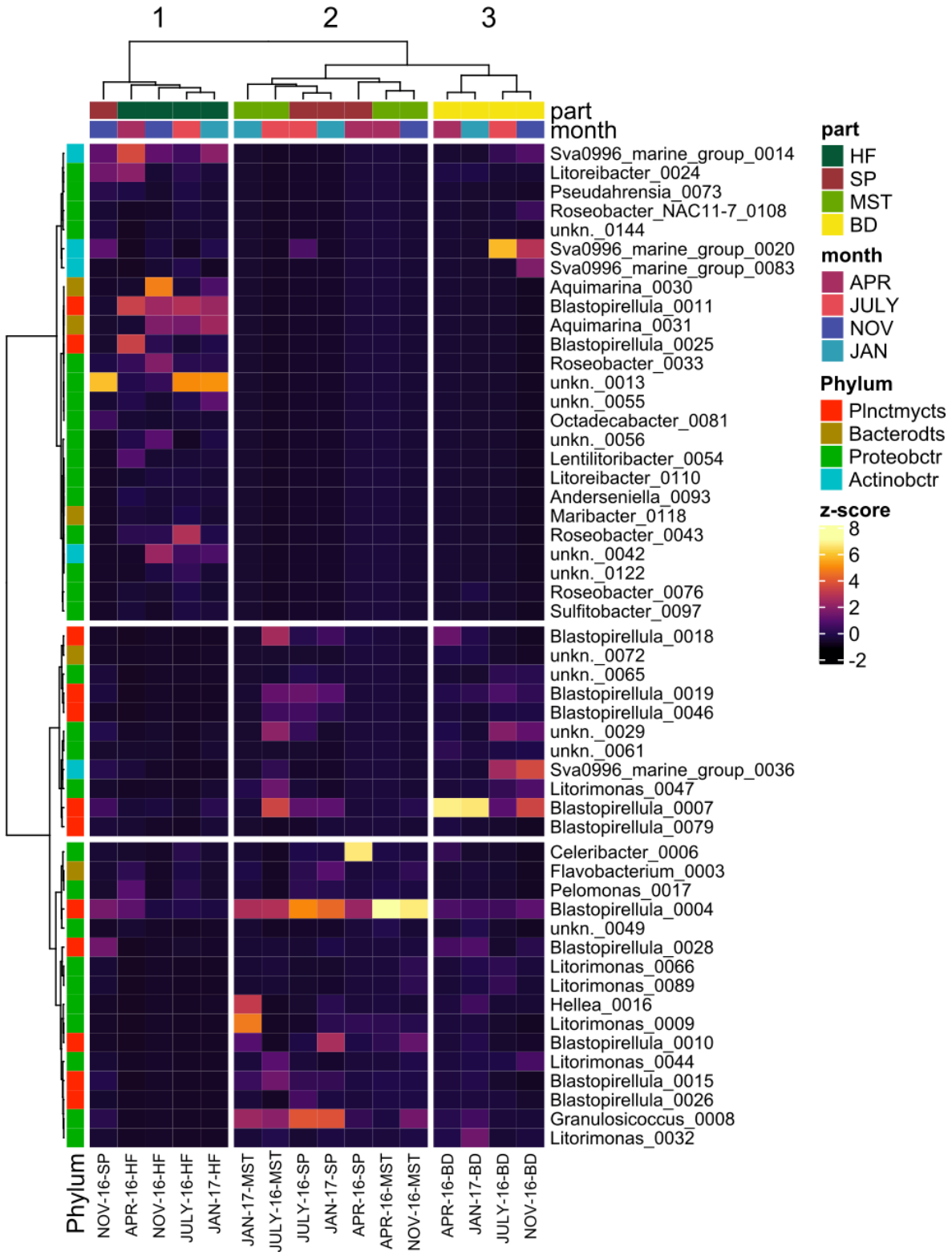

Figure 4.7: A heatmap of the 52 most abundant ASVs illustrating the broad differences in community composition between locations on *L. digitata*. Abundances are normalised to unit-variance (z-score transformation). Abbreviations: Proteobactr: *Proteobacteria*, Bacterodts: *Bacteroidetes*, Plnctmycts: *Planctomycetes*, Actinobctr: *Actinobacteria*, SprW: Spearman correlation with Ward-linkage, HF: holdfast, SP: stipe, MST: meristem, BD: blade, APR: April, JULY: July, NOV: November, JAN: January.

Clustering of samples by community dissimilarity (Bray-Curtis) corresponded well with the source of the sample. Clustering by part provides better separation than clustering by month, with both blade and holdfast presenting distinct communities while meristem and stipe cluster together (Figure 4.7), possibly reflecting the similar ecological niches they represent to epiphytes. Some genera (*Blastopirellula*, *Litorimonas*, Sva0996 marine group) are represented by multiple ASVs across the sampling regime; in particular *Blastopirellula* variants are found across the seaweed but show preference for specific locations on the algal body: *Blastopirellula*_0004 is most abundant at the stipe and meristem, while *Blastopirellula_0007* is more abundant on the seaweed blades, and *Blastopirellula* 0011 is most abundant at the holdfast, suggesting niche adaptations within the genus.

4.5 Discussion

Forest-forming kelp of the order *Laminariales* represent a significant nutritional source for several marine species and also support complex food webs in the marine ecosystem (Kang et al., 2008). Several authors have reported the biotechnological, food and agricultural applications of these macroalgae (Buck et al., 2006; Hou et al., 2015; Rupérez, 2002). However, a gap still exists in our current knowledge on the diversity and structure of seaweed-associated epiphytic bacteria. Over a decade ago, Staufenberger et al., (2008) reported the

phylogenetic analysis of bacteria associated with the four parts of *Saccharina lassitima* (formerly *Laminaria saccharina*) and how these populations differed at two seasons, using DDGE and 16S rRNA gene clone libraries. Bengtsson and co-workers also studied the seasonal and spatial variations between microbial populations found on the meristem and blade regions of *Laminaria hyperborean* (Bengtsson et al., 2010) while the bacterial populations associated with *Nereocystis luetkeana* and *Macrocystis pyrifera* blades and meristem have recently been reported (Weigel and Pfister, 2019). However, no such analysis has to date been carried out on *Laminaria digitata*. Thus the work presented here provides a comprehensive picture of how the surface-attached microbial population on all four parts of *Laminaria digitata* change over a 10 month period corresponding to four different sampling months, using next generation Illumina sequencing targeting the 16S rRNA V3-V4 gene region.

The brown algal bacterial community was characterized by the presence of *Proteobacteria*, *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Firmicutes* and *Verrucomicrobia* which are groups of bacteria that have previously been identified in related macroalgae (Bengtsson et al., 2010; Bengtsson and Øvreås, 2010; Ihua et al., 2019). This suggests that different macroalgal species harbour similar physiochemical properties suitable for the colonization of a broad range of generalist epiphytic bacteria (Florez et al., 2017; Lemay et al., 2018). Interestingly, only 32 ASVs (1.1 % of 2,908) were consistently found across all the different algal thallus parts and 103 ASVs (3.5 %) were common to the seasons. Tujula and co-workers previously reported that 60% of DDGE bands were shared amongst *Ulva australis* sampled across different seasons, and proposed the existence of a core community of bacterial species on algal surfaces (Tujula et al., 2010). However, taxonomic analysis in this present study demonstrates that *L. digitata* associated bacterial populations are unique to their morphological part and season. The lack of a rich core bacterial community present in this brown seaweed suggests that macroalgal-bacterial interactions are being undertaken by a broad range of bacterial groups capable of

performing similar functional roles which are required for the sustenance of the macroalgal ecosystem (Burke et al., 2011b; Naeem, 1998). This concept of functional redundancy implies that the recruitment of bacterial groups on macroalgae may depend on their functional competency, rather than taxonomy (Burke et al., 2011a), offering the algal-bacterial community structure a degree of resilience to disturbance (Naeem, 1998). However, the lottery hypothesis (Burke et al., 2011a; Burke et al., 2011b; Munday, 2004), further suggests that within a group of functionally competent species, the assembly and recruitment of the community can be stochastic in nature (Morrissey et al., 2019). Functional redundancy has been demonstrated in algal-associated bacterial communities and also in soil-derived microbial populations (Persiani et al., 2008; Yin et al., 2000). While a taxonomical core bacterial community could not be established in the epibacterial communities associated with *Ulva* species from different geographic locations, further genetic analysis revealed a functional core set of genes related to biofilm formation and in genes responding to environmental stimuli (Roth-Schulze et al., 2018).

Macroalgal-associated bacterial community profiles are shaped by a number of factors including geographic location, seasonal changes, host species, morphology and algal health (Chun et al., 2017; Mancuso et al., 2016; Weigel and Pfister, 2019). Functional and nutritional requirements within the different structural parts of an individual algal thallus are unique, thus creating a distinct morphological niche for each part. Stipes in *Fucus vesiculosus*, for example, have the lowest nitrogen assimilation rate, whereas *Laminaria* meristems are rich in nitrogen (Hurd et al., 2014). It might therefore be expected that the microbial populations associated with the different thallus parts would differ accordingly, as we observed here with *L. digitata* in this study, with significant differences being observed between the bacterial community profiles associated with the holdfast, stipe, meristem and blade regions of this brown seaweed (Figure 4.5). Spatial variation has also previously been reported in different parts of an

individual algal thallus or land plant (Bodenhausen et al., 2013; Weigel and Pfister, 2019). The leaf and root tissue of *Arabidopsis thaliana* for example have been shown to differ in their epiphytic bacterial composition (Bodenhausen et al., 2013) while greater OTU richness has been observed in the associated microbiota of the meristem of the kelp *Nereocystis luetkeana* than in the apical blade tissue (Weigel and Pfister, 2019).

Interestingly, in our study, the holdfast region in *L. digitata* was identified as the most diverse tissue whereas the meristem region was the least diverse. While this observation differs from Staufenberger et al., (2008) which reported the holdfast as the least diverse tissue in *S. latissima* with respect to its epiphytic bacterial community, it further demonstrates the uniqueness of bacterial communities associated with different seaweeds, including species with close phylogenetic relationships. The holdfast in kelp species is embedded in heterogeneous sediments which are rich in nutrients, due to the extensive settlement of chemicals and exudates from seaweeds and other surrounding marine species (Smith, 2000). These sediments which consist of organic and inorganic matter with various physical and chemical properties (Bodenhausen et al., 2013) are thus likely to be attractive for colonization by a wide range of diverse bacterial species.

Furthermore, while the community profiles of the holdfast, stipes, meristem and blade regions were shown to be remarkably different, taxonomic analysis in this study also revealed that each thallus part sampled harboured different microbial communities at four different months. These differences may arise as a result of seasonal variations in algal metabolites and exudates, as well as changes in seawater temperature; which as previously reported are known to influence algal surface chemistry and consequently the success of bacterial attachment (Lachnit et al., 2011; Steinberg et al., 1997). Coupled with environmental conditions, the life cycle of algal species which characterizes different seasons has also been suggested to modulate the structure and composition of the associated epibacterial population. For example, algal species are likely

to recruit fewer bacterial species when shedding old tissues as observed in *Laminaria hyperborea* (Bengtsson and Øvreås, 2010) and in the canopy-forming brown seaweed *Cystoseira compressa* (Mancuso et al., 2016).

In addition, macroalgal bacterial communities display patterns of succession to include more diverse and complex bacterial groups, as well as the increased prevalence of certain groups of bacteria, during the annual growth cycle of the algal host (Weigel and Pfister, 2019). Bengtsson and Øvreås (2010) for example, reported an increased relative abundance of *Planctomycetes* in *L. hyperborea* between July and September, when compared to other months, possibly due to the seasonal growth cycle of the seaweed. This phenomenon, together with the presence of organic exudates from the decaying seaweed could explain the prevalence of heterotrophic bacteria such as *Planctomycetes* (36 % mean relative abundance) which represented one of the most abundant phyla in April, July and November months in this study. Marine *Planctomycetes* are well known to degrade the sulfated-polysaccharide rich macroalgal cell walls (Glöckner et al., 2003), and are thus well adapted to growth on algal surfaces. The absence of peptidoglycan in the cell wall of *Planctomycetes* also offers these bacterial groups a potential competitive advantage with respect to an increased resistance to conventional algal antimicrobial defence mechanisms which target peptidoglycan-rich cell walls; and may also account for their abundance in algal-associated bacterial communities (Bengtsson and Øvreås, 2010; Cayrou et al., 2010; Lage and Bondoso, 2014). Likewise, the mineralization of organic compounds by heterotrophic *Planctomycetes* species may also provide nutritional benefits to marine seaweeds (Lage and Bondoso, 2014).

Overall, the potential factors which modulate macroalgal epibacterial communities can either exert independent influences or create a combination effect (Morrissey et al., 2019). Here, we observed a combination effect of both structural and positional differences in the algal thallus parts which result in unique morphological niches, and seasonal changes which contribute to

the functional requirements of the macroalgal ecosystem. These morphological and seasonal variations create distinct biogeographic signatures which drive the composition and structure of the bacterial populations associated with *Laminaria* digitata, and result in specific communities which lack a core population.

Supplementary Material

Table S4.1 showing the sample metadata and Tables S4.2 and S4.3 showing statistical analyses of differences between the epibacterial communities associated with different parts and seasons of *Laminaria digitata* samples respectively are available.

4.6 References

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Chapter 5: General Discussion

The aim of this work has been to investigate the composition and abundance of the bacterial communities which are associated with the two brown seaweeds, *Ascophyllum nodosum* and *Laminaria digitata*. We also aimed at examining their biotechnological potential, with respect to algal cell wall degrading enzymes which have an application in enzyme-assisted extraction technologies.

In chapters 2 and 3, our study focused on simulating an environment which would favour proliferation and allow the subsequent isolation of macroalgal polysaccharide degrading (MAPD) bacteria from *Ascophyllum nodosum* (**chapter 2**) and *Laminaria digitata* (**chapter 3**). Based on the proposed hypothesis that bacteria capable of degrading algal cell walls are likely to increase under nutrient-limiting conditions (Martin et al., 2015), our approach was to allow the macroalgal samples to slowly decay, in the absence of additional external nutrients which would allow bacterial growth. The primary focus of these chapters was to:

- Observe microbial population changes in the decaying seaweed
- Isolate MAPD bacteria from the seaweed both in its intact and decaying states
- Examine the biotechnological application of the MAPD bacteria, in particular their potential utility in enzyme-assisted extraction (*Ascophyllum nodosum* only; chapter 2)

To achieve the above mentioned objectives, both metagenomic and culture based approaches were employed, the latter which included the novel iChip bacterial isolation method (Nichols et al., 2010). From our findings in **chapter 2**, the metagenomic communities associated with *Ascophyllum nodosum* comprised of bacteria which belong to 22 different phyla, the most abundant being *Proteobacteria, Bacteroidetes, Spirochaetae, Firmicutes, Planctomycetes, Verrucomicrobia, Actinobacteria* and *Lentisphaerae* (Figure 2.1). However only four bacterial phyla (*Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria*) were identified in the

cultivable surface microbiota of the brown seaweed (Figure 2.2). Similarly, our analysis reported in **chapter 3** revealed that some bacterial groups present in the metagenomic communities associated with *Laminaria digitata* were not recovered in the cultivable bacterial population of the macroalga. This disparity highlights the limitations of culture-based approaches in analysing the presence and ultimately the biotechnological potential of microbial populations from different environments. Despite the use of numerous bacterial isolation methods which can involve the use of diverse nutrient media, a wide range of incubation temperatures, together with extended incubation times (Alain and Querellou, 2009), it is well established that only less than 1% of bacteria in any environmental sample can be successfully cultivated and subsequently isolated (Vartoukian et al., 2010). The increase in next generation sequencing strategies in the last decade such as 454 pyrosequencing (Quince et al., 2009), Illumina sequencing (Meyer and Kircher, 2010), Ion Torrent (Brown et al., 2013) and PacBio (Rhoads and Au, 2015), has resulted in the provision of relatively cheap and affordable means to advance microbial ecological studies through the analysis of metagenomic DNA samples from different ecosystems. Current genomic tools have thus helped to provide fresh insights and made an immense contribution to extending our view of the previously underestimated microbial world. Through the use of improved sequencing technologies, studies such as the Earth Microbiome project (Thompson et al., 2017) which aims at analysing approximately 200,000 environmental samples (Gilbert et al., 2014) have been possible. The scientific community has also been able to examine the human microbiome (Turnbaugh et al., 2007) as well as several factors which influence the gut microbiota (Claesson et al., 2012; O'Toole and Jeffery, 2015; Xu and Knight, 2015). There is currently however a bottleneck in the discovery process as a result of the very high levels of biological data being generated which require ample time and core bioinformatics skills to decipher and translate into applicable knowledge (Kulkarni and Frommolt, 2017; Pop and Salzberg, 2008).

Our results from chapters 2 and 3 revealed that in both seaweeds, the physiological state of the seaweed had an impact on its associated epibacterial populations, with distinct communities being observed in both the intact and decaying samples. Microbial communities associated with seaweeds as well as other environmental samples are well known to be modulated by specific environmental conditions. In particular, the condition of the host – whether healthy or stressed - has been identified as making a significant contribution to the structure of the associated bacterial communities. Continental scale variations in the bacterial communities on *Ecklonia radiata*, for example, were observed to be strongly related to the algal host healthy or bleached state (Marzinelli et al., 2015). Similarly, the epibacterial populations found on the healthy red alga *Delisea pulchra* were distinct from those present on the bleached counterpart which was demonstrated to display lower levels of antibacterial activities (Campbell et al., 2011). While the intricate processes leading to algal decay are currently not well established, previous studies have shown that certain changes in the microbial structure may arise as a result of loss of the physiological integrity of macroalgae which consequently influence the immediate microcosm. Zhu and co-workers (Zhu et al., 2013) have in fact studied the effect of algal decay on the nutrient levels of the water sediment in China and their findings suggested that the decomposition of marine algae results in decreased dissolved oxygen levels, together with increased ammonium and orthophosphate levels. Changes in nutrient concentration levels were also monitored in decaying *Cladophora* mats (Chun et al., 2017) and revealed similar conclusions. In addition to these observed changes in nutrient profiles, Chun and co-workers (Chun et al., 2017) further demonstrated a shift in the structure of *Cladophora*-associated microbial communities following decay, towards a population which was abundant in potentially pathogenic bacteria.

Furthermore, the results of the study conducted and presented in chapter 2 and chapter 3 support the current understanding that the assemblage of macroalgal associated microbial communities

can be host specific. We observed clear differences between the epibacterial communities which are associated with the two brown seaweeds, *Ascophyllum nodosum* and *Laminaria digitata*. Overall, *Ascophyllum nodosum* communities were found to be more diverse, with 22 different phyla being identified in the metagenomic datasets, for example, whereas only six phyla were present in *Laminaria digitata*. In addition to the species-specific surface chemistry which may influence the attachment of macroalgal bacteria (Lachnit et al., 2011; Singh and Reddy, 2014), the biochemistry and metabolome of algal species may also contribute to the variations observed as each seaweed produces a unique cocktail of chemicals which can either attract or repel certain bacteria. Recent work by Kessler et al., (2018) demonstrates that macroalgal interactions are mediated by specific chemotactic signalling molecules which enables cross talk between bacteria and their algal hosts. Dimethylsulfoniopropionate (DMSP) released by *Ulva mutabilis* for example has been shown to attract the colonization of *Roseovarius* sp. which converts the DMSP osmolyte into methanethiol (MeSH) and dimethylsulphide (DMS) (Kessler et al., 2018). These algal chemical signals are also recognised and interpreted by bacteria as an indication of the availability of food and other resources required for their growth. These finding suggest that further studies on the different metabolic profiles of seaweed species are required to not only deepen our understanding of the physiology and ecology of marine algae, but also to help contribute to a gaining a wider perspective on macroalgal interactions (Gravot et al., 2010).

Beyond observing the bacterial population changes in the decaying brown seaweeds, our study also focused on examining the functional characteristics of the bacterial species present. Screening the cultivable surface microbiota for the production of enzymes with activity against selected algal polysaccharides, revealed that approximately 7% of the bacterial population isolated from the decaying *Ascophyllum nodosum* (chapter 2) produced at least one of the MAPD enzymes (lichenase, pectinase, cellulase) under the conditions tested; whereas none of

the bacterial isolates from the intact macroalgae were found to produce any of these enzymes. Likewise, over 50% of the bacterial population which were isolated from decaying *Laminaria digitata* were identified as MAPD bacteria, compared to less than 5% of total bacterial isolates prior to decay (chapter 3). These results demonstrate that: only a minor fraction of the epibacterial populations associated with intact/uncompromised macroalgae are MAPD producers. This may arise as an evolutionary mechanism to limit algal damage which results from degraded cell walls. Other studies on intact macroalgae also report that MAPD bacteria constitute a minority of the overall algal associated bacterial communities present (Martin et al., 2015). Also, our results demonstrate that the prevalence of MAPD bacteria on algal surfaces increases when the algae become weakened such as under conditions when they decay. Marine algae have been reported to generate up to half of the global net primary production which is equivalent to 104.8 pentagram of carbon on annual basis (Field et al., 1998) and the conversion of marine algal organic matter to low molecular weight compounds by heterotrophic bacteria has been described as one of the "largest and fastest catabolic biotransformations on earth" (Hehemann et al., 2014). By ensuring photosynthetically fixed carbon is returned back to the atmosphere, marine bacteria are critical players in global carbon cycling (Martin et al., 2015; Michel et al., 2006). It is thus possible that the increased prevalence of MAPD bacteria during decay may contribute to algal biomass recycling and function as an efficient mechanism of carbon release.

From both brown seaweeds, we identified a total of 55 bacterial strains mainly identified as either *Bacillus* or *Pseudoalteromonas* species which produced more than one of the algal cell wall degrading enzymes which were screened for in this study (Table S2.1, Table S3.1). This suggests that these MAPD bacteria possibly produce a wide range of MAPD enzymes which may work in synergy to effectively degrade the algal cell wall which comprises of a diverse range of polysaccharides. Perhaps, some of these active isolates may produce multifunctional

enzymes (MFEs) which can perform several autonomous functions (Cheng et al., 2012). A precedent for the possible presence of MFEs in our enzyme active bacterial isolates is evident from recent work conducted in our laboratory on a large insert fosmid metagenomic library which was created using metagenomic DNA extracted from *Laminaria digitata* (Stephen Jackson, personal communication). The library was screened for enzymatic functions and one clone displayed activities on starch as well as on carrageenan. The active insert was sequenced and it was determined that both activities were from a single enzyme. This large protein, comprising 423 amino acids, showed high homology to the phosphomannomutase/phosphoglucomutase gene (algC). The enzyme was similar to those found in the halophilic or halotolerant species *Cobetia marina*, *Halomonas* spp. and *Virgibacillus* spp. and *Chromohalobacte*r spp. Such multiple enzymatic activities displayed by MFEs are independent of multiple RNA splice variants, gene fusions or pleiotropic effects (Huberts and van der Klei, 2010) but are believed to result in some cases as a result of substrate promiscuity (Khersonsky et al., 2006). Under different conditions which require adaptation, MFEs allow microbes to switch their biochemical signalling pathways and expand their biological function with minimal changes in energy demand and without the cost of increasing their genome size (Jeffery, 1999; Jeffery, 2004). Multifunctional enzymes such as Amy63 which displays potent alpha-amylase, agarase and carrageenanase activities, have previously been isolated from marine bacteria (Liu et al., 2016). To identify the true potential of these MAPD bacteria and possibly obtain novel enzymes with improved biochemical properties, further metagenomic-based approaches would need to be employed. One possible method would be the construction of plurigenomic libraries (comprising of multiple genomic DNA) from some of our enzyme-active bacterial isolates. Selection of bacterial isolates previously known to produce enzymes of interest for clone libraries offers the advantage of a more streamlined and focused approach to the search for novel enzymes. Functional screening of

such plurigenomic libraries and subsequent data mining of positive clones can prove to be an effective approach for the identification of completely new algal polysaccharidase encoding genes as previously reported (Martin et al., 2016). It is also important to highlight here that up to 40% and 20% of the metagenomic communities associated with *Ascophyllum nodosum* (chapter 2) and *Laminaria digitata* (chapter 3) respectively, remained unclassified according to the SILVA taxonomy database employed (Quast et al., 2012), with the majority belonging to the decaying samples. These unidentified sequences from decaying macroalgae may also represent potentially novel bacteria species which could possess a wide range of metabolites or enzymes with useful biotechnological applications. Therefore, in addition and complementary to the culture-based methods already employed in this study, the additional construction of metagenomic libraries from the decaying seaweeds could also lead to the discovery of novel microbial enzymes which display unique biochemical properties from these macroalgal microbiome samples.

In addition, one of the major highlights of **chapter 2** was the application of the crude supernatants obtained from three *A. nodosum* derived bacterial isolates (ANT0_A6, IC18_D5, IC18_D7) on the enzyme-assisted extraction of phenolics from *Fucus vesiculosus* which resulted in a 11-13% extraction yield, which was comparable to commercially available enzymes (Figure 2.3). Firstly, this result emphasizes the well-established finding that algal-associated microbial communities are enriched with bacteria which produce enzymes that are potentially useful for biotechnological applications. *Fucus vesiculosus* is a brown seaweed which is related to *Ascophyllum nodosum* and which has previously been reported to comprise of bacterial populations which are similar to the communities identified in this study (Pugovkin et al., 2016; Stratil et al., 2013). While bacterial populations associated with macroalgae can be host-specific as demonstrated in previous studies (Lachnit et al., 2011; Morrissey et al., 2019), certain generalist epiphytes (Florez et al., 2017; Lemay et al., 2018) which can colonize

a wide range of algal species and perform similar ecological roles have also been reported. Thus it is not surprising perhaps, that the enzymes produced by the *Ascophyllum* derived bacterial isolates were also capable of degrading the *Fucus* cell wall to facilitate the extraction of phenolics from the alga.

It is plausible that the crude bacterial extract from the three bacterial strains comprises of a number of as yet unidentified algal cell wall degrading enzymes which may have acted in combination to elicit the extraction yield observed. Much work remains to be done to further characterize this cocktail of enzymes contained within the bacterial supernatants. Firstly, the various native enzymes would need to be purified from the crude extract. Subsequently, zymogram analyses and plate-based activity assays of a number of algal polysaccharidases at wide temperature and pH ranges, together with enzyme activity quantification would need to be performed. Where necessary, MALDI-TOF/TOF mass spectrometry could be employed to begin to elucidate the structures and potential novelty of the enzymes (Liu et al., 2016).

Having observed the diversity of the bacterial populations associated with *Ascophyllum nodosum* (chapter 2) and *Laminaria digitata* (chapter 3) and examining their biotechnological applications, we were curious to further investigate these brown seaweeds. In particular, considering that the *Laminaria digitata* results presented in chapter 3 were only from the meristem region of the macroalga, we were interested in examining the microbial diversity and composition associated with other sections of the algal thallus (stipe, holdfast and blade). While other studies have reported variations between different parts of an algal thallus (Bengtsson et al., 2010; Staufenberger et al., 2008; Weigel and Pfister, 2019), including related seaweeds such as *Saccharina lassitima*, no such work has to date been undertaken on *Laminaria digitata*. Therefore we reasoned that such an approach would significantly contribute to and expand the current body of knowledge on the microbial ecology of brown seaweeds. Furthermore, we decided to observe how the microbial communities associated with each of the thallus part

would respond to seasonal changes, thus samples were collected over a period of ten months. We expect that the results from this study would provide useful insight to inform the scientific community as well as commercial seaweed harvesters as to which part of the alga and season is likely to be associated with an increase in microbial diversity or harbour specific bacterial groups of interest with desired characteristics such as algal cell wall degrading activities with potential application in enzyme-assisted extraction technologies and other biotechnological applications.

In **chapter 4**, the microbial populations associated with the holdfast, stipe, meristem and blade regions of *Laminaria digitata* which were each sampled in April 2016, July 2016, November 2016 and January 2017 were investigated using Illumina sequencing of the 16S rRNA genes. The main results indicated that morphological and seasonal variations have a strong effect on the associated bacterial communities of *Laminaria digitata*. In addition, the meristem and stipe samples appear to be more closely related in their microbial structure, whereas holdfast and blades remain separate and distant. The holdfast and November samples represented the most diverse region of the seaweed and sampling month respectively, while it was clear that the algal associated epibionts were specific and lacked a core population, with the shared sequences representing only less than 3.5 % of the population.

As demonstrated in our previous studies (chapters 2 and 3), a culture independent based metagenomic approach can provide a very comprehensive picture on the associated microbial communities. Our study together with several other studies have resulted in the generation of metagenomic data on algal associated microbiomes (Mancuso et al., 2016; Weigel and Pfister, 2019). However, the current challenge lies in the functional characterization of these complex and diverse bacterial communities. Further investigations to unravel the functional microbiome of macroalgae using high-resolution bioinformatics methods such as predictive functional profiling of the associated microbial communities with marker gene sequences (Langille et al.,

2013) involved in cross-domain signalling between bacteria and their algal host, carbon and nitrogen cycling, amongst many ecological functions (Singh and Reddy, 2016) need to be undertaken to shed further light in our overall understanding of the physical, biological and chemical factors which drive epibacterial communities on individual algal species. Notwithstanding this however, given the variability of the marine environment, it is likely that these seaweed-bacterial interactions may differ, thus, transcriptomics, metabolomics and proteomics studies will ultimately need to be performed in parallel together with culture based approaches; to gain the insights necessary to harness the biotechnological potential of algal-bacterial relationships under a broad range of environmental conditions.

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6.0 Publications and Communications

6.1 Publication list

Ihua, M. W., Guihéneuf, F., Mohammed, H., Margassery, L. M., Jackson, S. A., Stengel, D. B., Clarke, D. J. & Dobson, A. D. (2019). Microbial Population Changes in Decaying *Ascophyllum nodosum* Result in Macroalgal-Polysaccharide-Degrading Bacteria with Potential Applicability in Enzyme-Assisted Extraction Technologies. *Marine Drugs,* 17**,** 200 **(published)**

Ihua, M. W., Fitzgerald, J.A., Guihéneuf, F., Jackson, S. A., Stengel, D. B., Clarke, D. J. & Dobson, A. D. (2019). Morphological niches and seasonal variations drive the structure and composition of *Laminaria digitata* associated bacterial communities. *Front Microbiol* **(submitted)**

Ihua, M. W., Stengel, D. B., Clarke, D. J. & Dobson, A. D. (2019). Microbial population changes in decaying *Laminaria digitata* result in an enrichment in bacteria with enzymes capable of algal cell wall degradation. **(manuscript in review)**

6.2 Oral Presentations

"Decaying *Ascophyllum nodosum* as a source of algal cell wall degrading enzymes, with potential utility in enzyme-assisted extraction technologies" **Irish Algal Researchers Conference (Galway, Ireland) 2018** and **Microbiology Annual Society Conference (Belfast, UK) 2019**

"Seaweeds are awesome, let's get more juice out of them". **Pint of Science Festival (Cork, Ireland) 2019**

6.3 Poster Presentations

Ihua, M. W., Guihéneuf, F., Mohammed, H., Margassery, L. M., Jackson, S. A., Stengel, D. B., Clarke, D. J. & Dobson, A. D. "Decaying *Ascophyllum nodosum* as a source of algal cell wall degrading enzymes, with potential utility in enzyme-assisted extraction technologies" **Environ Conference (Cork, Ireland) 2018**

Ihua, M. W., Guihéneuf, F., Mohammed, H., Margassery, L. M., Jackson, S. A., Stengel, D. B., Clarke, D. J. & Dobson, *A. D. "*Bacteria from decaying *Ascophyllum nodosum* as a source of algal cell wall polysaccharidases with potential use in enzyme-assisted extraction technology" **ISME 17 (Leipzig, Germany) 2018**

Stengel, D. B., Ihua, M. W., Guihéneuf, F., Mohammed, H., Margassery, L. M., Jackson, S. A., Clarke, D. J. & Dobson, A. D. "Decaying *Ascophyllum nodosum* as a source of algal cell wall degrading enzymes, with potential utility in enzyme-assisted extraction technologies" **, European Phycological Congress (Zagreb, Croatia) 2019**

Ihua, M., Dobson, A. and Stengel, D., 2019. Metagenomic analysis reveals significant seasonal variations in the epiphytic bacterial communities associated with different parts of the brown seaweed *Laminaria digitata*. **Microbiology Annual Society Conference (Belfast, UK) 2019**

Supplementary information

7.1 Chapter 2

Table S2.1: *Ascophyllum nodosum* associated bacterial isolates, their closest BLAST relative and observed enzymatic activities. Bacterial strains were examined for their hydroxyethyl cellulose (HE-cellulase), lichenase and pectinase activities. Enzymatic activity is indicated by a (+) sign while a (-) sign indicates that no enzymatic activity was observed under the conditions tested

Sample ID	Top BLAST hit	Identity (%)	Algal cell wall polysaccharide degrading activities		
			HE-cellulase	Lichenase	pectinase
AN218_A2	*Bacillus safensis* strain Rb1S1	100	-	+	-
AN218_H5	*Bacillus* sp. M101(2010) strain M101	100	+	+	-
AN225_A5	*Bacillus altitudinis* strain CT10	99	-	+	-
AN225_A11	*Bacillus licheniformis* strain HQB814	99	+	-	+
AN225_B8	*Bacillus licheniformis* strain AG-06	100	-	-	+
AN225_B9	*Bacillus licheniformis* strain ST7	99	-	-	+
AN225_C1	*Bacillus pumilus* strain ASpB9	99	-	+	-
AN225_C7	*Bacillus aerius strain* APBSMLB109	99	-	+	-
AN225_C11	*Bacillus* sp. 11RB3	99	-	+	+
AN225_D1	*Bacillus licheniformis* strain APBSWPTB167	100	+	-	+
AN225_D4	*Bacillus subtilis* strain HDXJ04	99	+	+	+
AN225_D6	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN225_E1	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN225_E6	*Bacillus licheniformis* strain JMB003	99	-	-	+
AN225_E7	*Bacillus licheniformis* strain V24	100	-	-	+
AN225_E8	*Bacillus licheniformis* strain V24	100	-	-	+
AN225_E9	*Bacillus* sp. strain SKS7	99	-	-	+

AN225_E10	*Bacillus licheniformis* strain KB102	99	-	-	+
AN225_E11	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN225_F6	*Bacillus* sp. strain 703	100	-	-	+
AN225_F9	*Bacillus* sp. strain C60	99	-	+	
AN225_F12	*Bacillus licheniformis* strain V24	100	+	-	+
AN225_G2	*Bacillus* sp. strain SKS7	100	+	-	+
AN225_G3	*Bacillus* sp. (in: Bacteria) strain VI/7	100	-	-	+
AN225_G6	*Bacillus subtilis* strain AKKVG-2-18	100	-	+	+
AN225_G8	*Bacillus* sp. (in: Bacteria) strain VI/7	100	-	-	+
AN230_A10	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN230_B4	*Bacillus* sp. strain SKS7	100	+	-	+
AN230_B11	*Bacillus mycoides* strain LBUM203	99	+	-	+
AN230_D9	*Bacillus licheniformis* strain V24	100	+	-	+
AN230_D11	*Bacillus licheniformis* strain V24	100	+	-	+
AN230_E3	*Bacillus* sp. (in: Bacteria) strain V52	100	-	-	+
AN230_E4	*Bacillus* sp. Ph_25A	100	-	+	-
AN425_D9	*Bacillus* sp. strain CZL003	100	+	+	+
AN425_D11	*Bacillus licheniformis* strain 8B-B92	99	+	-	+
AN425_D12	*Bacillus* sp. strain SKS7	100	+	-	+
AN425_E4	*Bacillus* sp. strain BS155	100	+	+	+
AN425_G7	*Bacillus* sp. strain BS155	100	+	-	+
AN618_A1	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN618_A2	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN618_B10	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN618_D11	*Bacillus pumilus* strain ASpB9	100	+	+	-
AN618_H4	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN625_A10	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN625_D7	*Bacillus pumilus* isolate TD22	100	-	+	-
AN625_G10	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN630_A12	*Bacillus hwajinpoensis* strain 16E11	99	-	+	-
AN630_D1	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN630_D2	*Bacillus pumilus* strain ASpB9	100	-	+	-

AN630_G12	*Bacillus pumilus* strain ASpB9	100	-	+	-
AN630_H8	*Bacillus safensis* strain Rb1S1	100	-	+	-
IC18_D5	*Vibrio oceanisediminis* strain S37	98	-	-	+
IC18_D6	*Vibrio anguillarum* strain INTA11	100	-	-	+
IC18_D7	*Vibrio anguillarum* strain X0906	99	-	-	+
IC18_D8	*Vibrio oceanisediminis* strain S37	99	-	-	+
IC18_D9	*Vibrio anguillarum* strain X0906	99	-	-	+
IC18_E2	*Vibrio oceanisediminis* strain S37	98	-	-	+
IC18_E6	*Vibrio anguillarum* strain KAP1	100	-	-	+
IC18_E7	*Vibrio oceanisediminis* strain S37	99	-	-	+
IC18_E8	*Vibrio anguillarum* strain INTA11	100	-	-	+
IC25_C11	*Micrococcus yunnanensis*	100	-	-	+
IC25_F10	*Micrococcus yunnanensis*	100	-	-	+

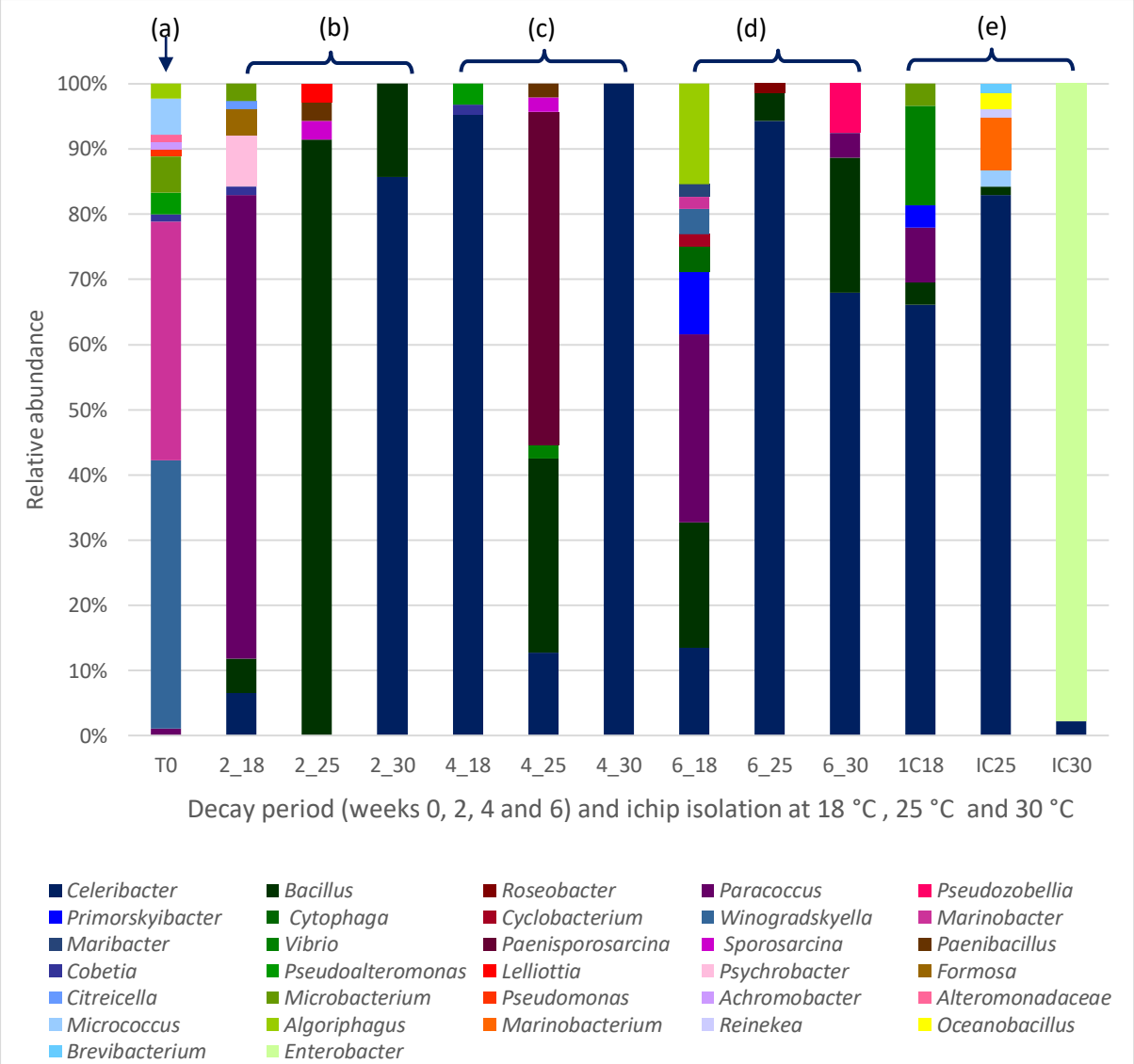

Figure S2.1

Relative abundances at genus level of bacteria associated with the cultivable surface microbiota of (a) intact *Ascophyllum nodosum* and decaying *Ascophyllum nodosum* at 2, 4 and 6 weeks of decay at (a) 18 °C; 2_18, 4_18, 6_18 (b) 25 °C; 2_25, 4_25, 6_25 (c) 30 °C; 2_30, 4_30, 6_30 which were obtained by maceration culture isolation method and (e) obtained by ichip culture isolation method. 16S rRNA gene sequences were obtained from the bacterial isolates and taxonomic analyses were performed. The relative distribution of phyla in each group is represented as a percentag

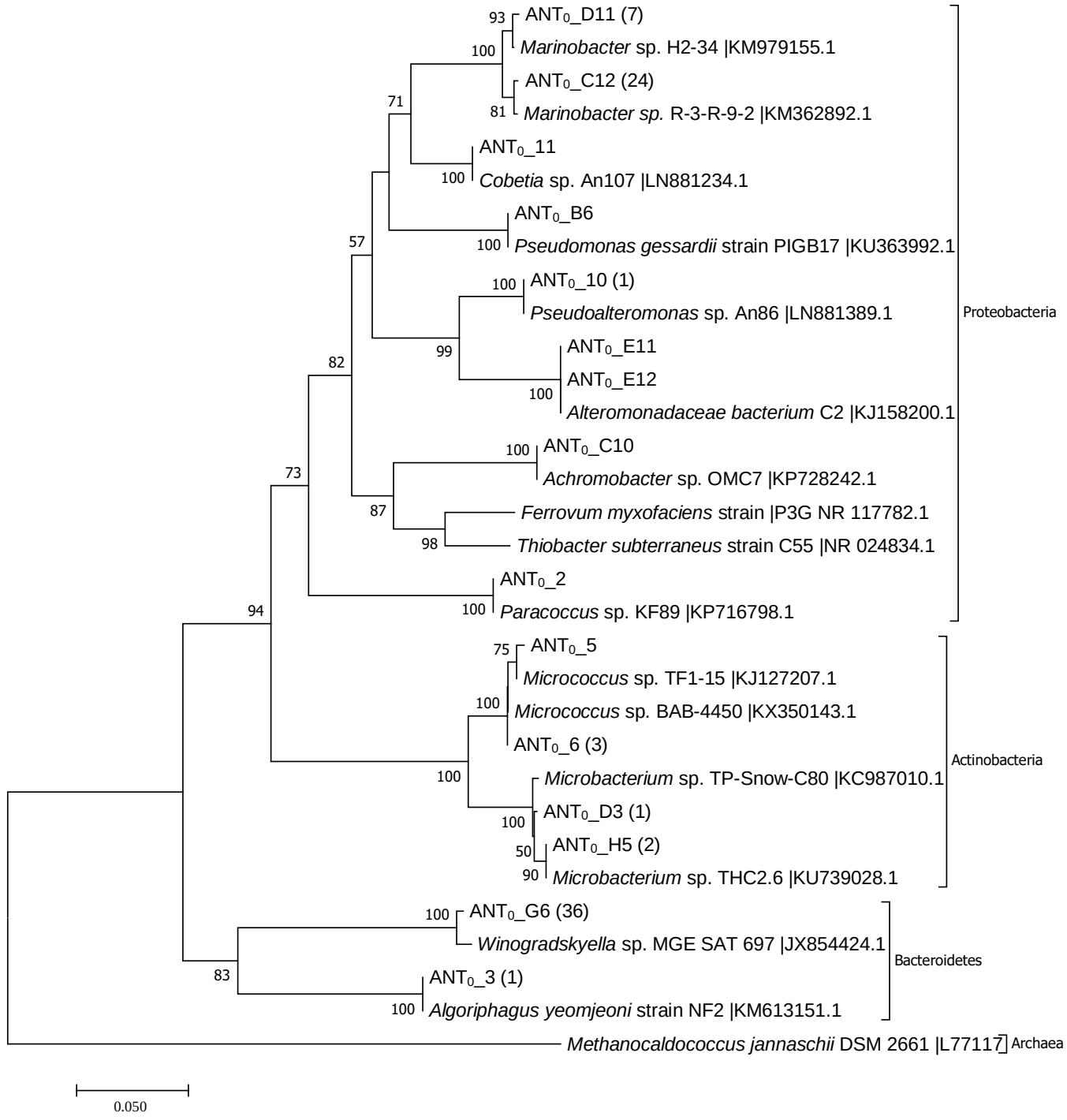

Figure S2.2

Neighbor-joining phylogenetic tree representing bacterial phyla cultured from *Ascophyllum nodosum* sample before induced decay (T_0). The evolutionary relationships of each phylum identified are shown with reference sequences from NCBI included. This phylogenetic analysis was performed using single representative 16S rDNA sequences from each group identified by

Fastgroup program. The number of similar sequences represented by each sequence is shown in brackets. This tree was drawn using MEGA program (version 7) and bootstrapping percentages (1000 replicates) above 50% are shown

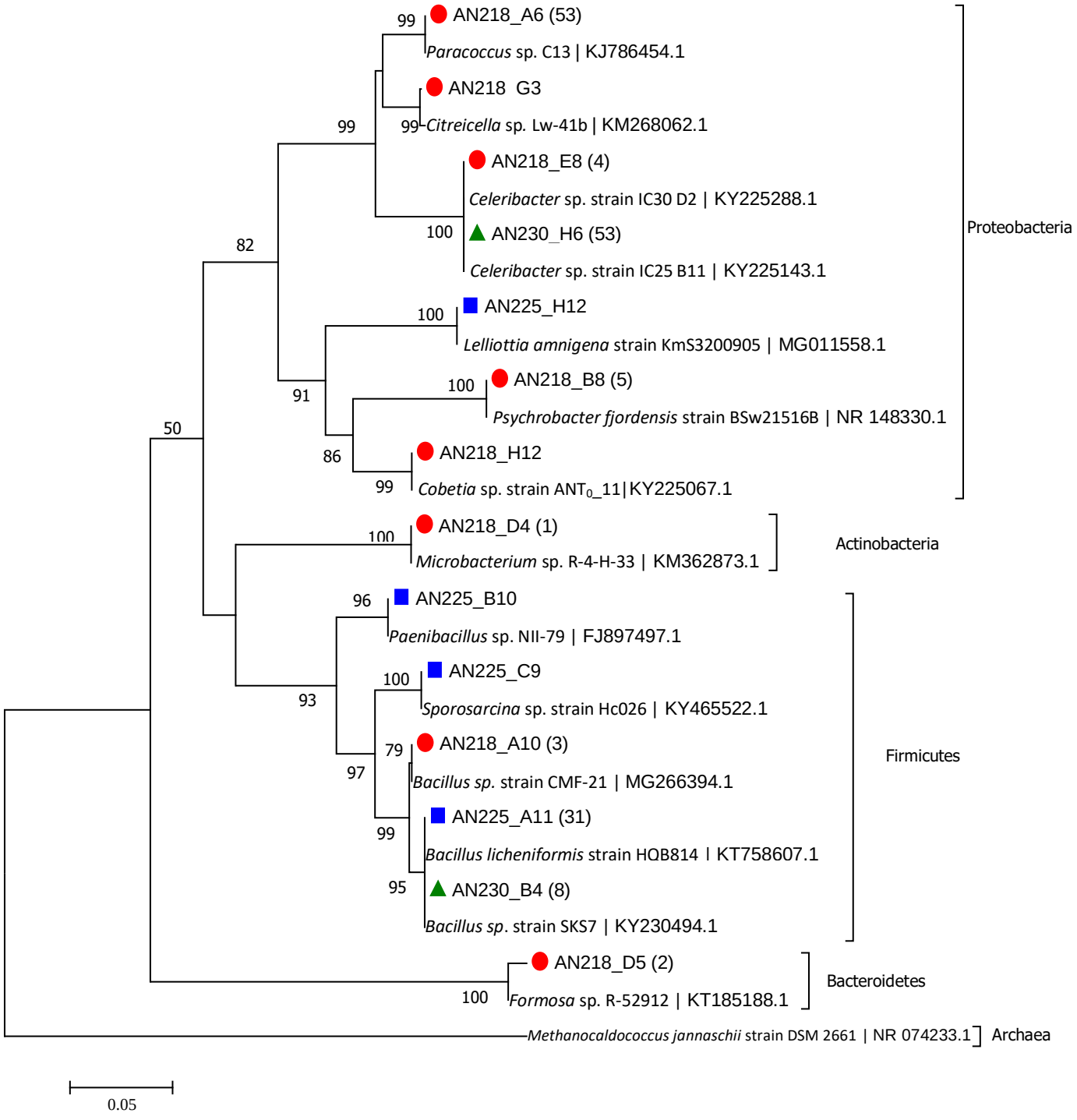

Figure S2.3

Neighbor-joining phylogenetic tree representing bacterial phyla cultured from *Ascophyllum nodosum* sample at week 2 of induced decay from ●18 °C, ■25 °C and ▲30 °C. The evolutionary

relationships of each phylum identified are shown with reference sequences from NCBI included. This phylogenetic analysis was made using single representative 16S rDNA sequences from each group identified by Avalanche NextGen Workbench version 2.30. The number of similar sequences represented by each sequence is shown in brackets. This tree was drawn using MEGA program (version 7) and bootstrapping percentages (1000 replicates) above 50% are shown

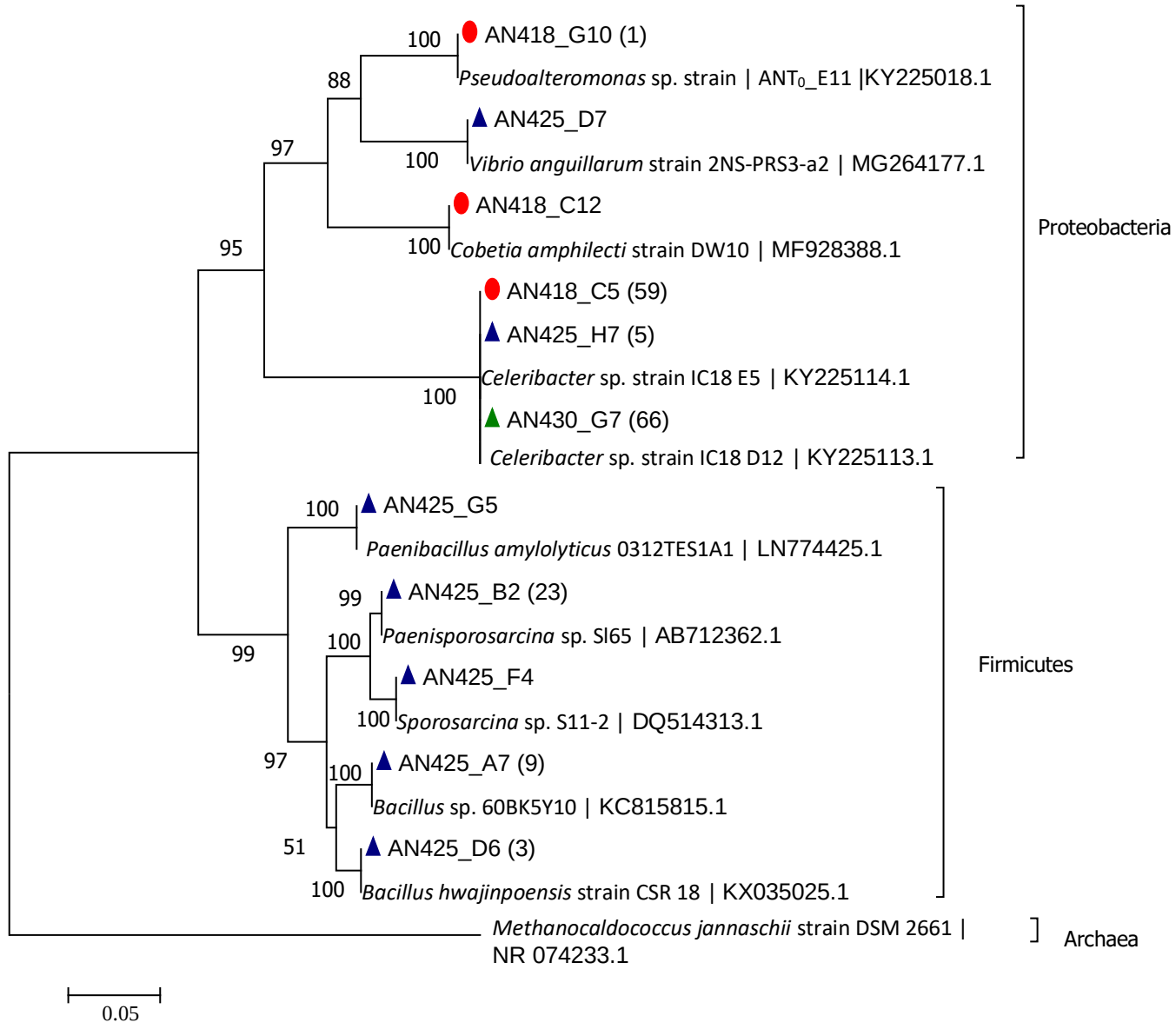

Figure S2.4

Neighbor-joining phylogenetic tree representing bacterial phyla cultured from *Ascophyllum nodosum* sample at at week 4 of induced decay from ● 18 ºC, ■ 25 ºC and ▲30 ºC. The evolutionary relationships of each phylum identified are shown with reference sequences from NCBI included. This phylogenetic analysis was made using single representative 16S rDNA sequences from each group identified by Avalanche NextGen Workbench version 2.30. The

number of similar sequences represented by each sequence is shown in brackets. This tree was drawn using MEGA program (version 7) and bootstrapping percentages (1000 replicates) above 50% are shown.

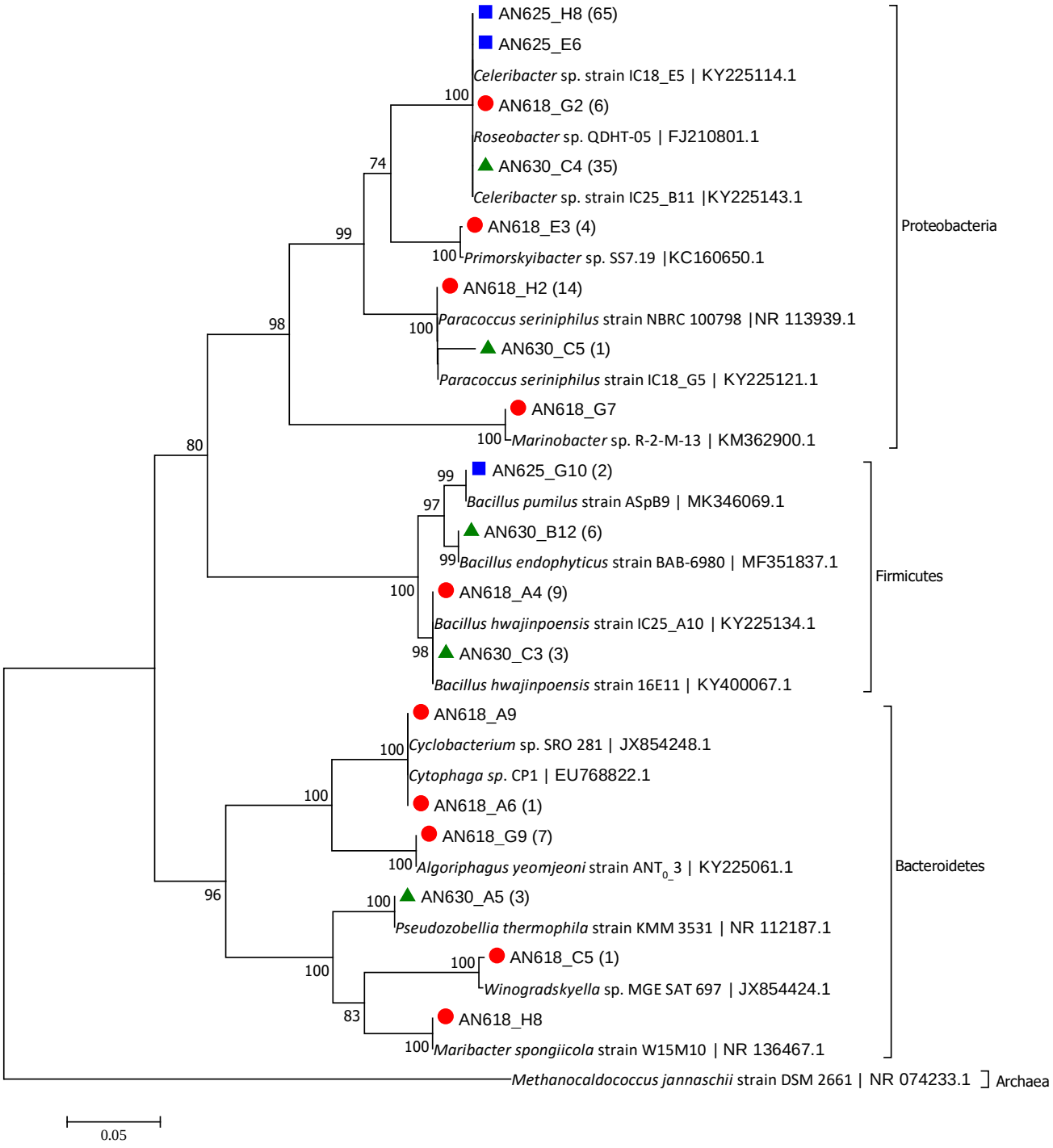

Figure S2.5:

Neighbor-joining phylogenetic tree representing bacterial phyla cultured from *Ascophyllum nodosum* sample at at week 6 of induced decay from ● 18 ºC, ■ 25 ºC and ▲ 30 ºC. The evolutionary relationships of each phylum identified are shown with reference sequences from

NCBI included. This phylogenetic analysis was made using single representative 16S rDNA sequences from each group identified by Avalanche NextGen Workbench version 2.30. The number of similar sequences represented by each sequence is shown in brackets. This tree was drawn using MEGA program (version 7) and bootstrapping percentages (1000 replicates) above 50% are shown.

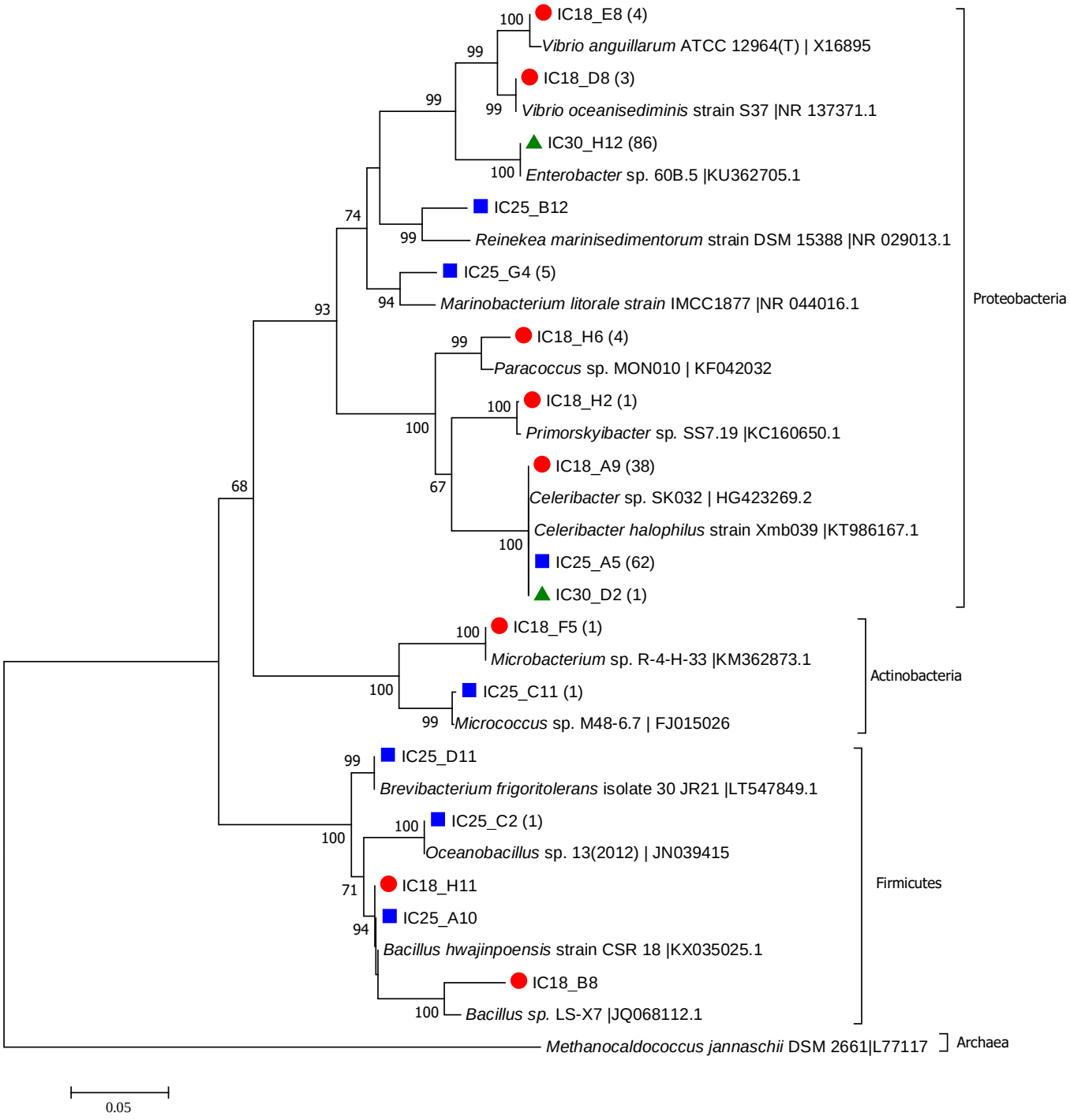

Figure S2.6

Neighbour-joining phylogenetic tree representing bacterial phyla cultured from 18 °C, 25 °C and 30 °C using the ichip device. The evolutionary relationships of each phylum identified

are shown with reference sequences from NCBI included. This phylogenetic analysis was performed using single representative 16S rDNA sequences from each group identified by Fastgroup. The number of similar sequences represented by each sequence is shown in brackets. This neighbor joining tree was drawn using MEGA program (version 7) and bootstrapping percentages (1000 replicates) above 50% are shown

7.2 Chapter 3

Table S3.1

Cultivable surface microbiota of intact (LDT0) decaying *Laminaria digitata* at 20 ˚C (LD20), 25 ˚C (LD25) and 30 ˚C (LD30); their closest BLAST relative and observed enzymatic activities. Bacterial strains were examined for their hydroxyethyl cellulose (HE-cellulase), lichenase and pectinase activities. Enzymatic activity is indicated by a (+) sign while a (-) sign indicates that no enzymatic activity was observed under the conditions tested

Sample ID	Top BLAST hit	Identity (%)	Algal cell wall polysaccharide degrading activities		
			Lichenase	pectinase	HE-cellulase
LDT0D8	*Paenibacillus* sp. strain JZ16	100	+	+	+
LD20A1	*Pseudoalteromonas translucida* strain BBCC2847	99	+	-	+
LD20A2	*Pseudoalteromonas* sp. strain QY-1	100	+	-	+
LD20A5	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	+
LD20A11	*Pseudoalteromonas nigrifaciens* strain HH-4	100	+	-	+
LD20B4	*Pseudoalteromonas* sp. strain BSw22120	100	+	-	+
LD20B8	*Pseudoalteromonas* sp. strain DZ-01-01-aga	99	+	-	+
LD20B9	*Pseudoalteromonas translucida* strain BBCC2847	99	+	-	+
LD20B12	*Pseudoalteromonas distincta* strain BBCC2856	100	+	+	-
LD20C2	*Pantoea* sp. strain ICMP 20864	100	+	-	-
LD20C5	*Bacillus altitudinis* strain FL103	100	+	-	-
LD20C10	*Bacillus zhangzhouensis* strain D61	100	+	+	-
LD20C11	*Bacillus zhangzhouensis* strain D61	100	+	-	-
LD20C12	*Pseudoalteromonas* sp. strain BSw22140	99	+	-	-
LD20D2	*Halomonas* sp. ARCTIC-P3	100	-	+	-
LD20D4	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	+
LD20D5	*Bacillus aerius* strain UB02	100	+	-	-
LD20D9	*Pseudoalteromonas* sp. strain BSw22140	100	+	-	+
LD20D10	*Psychrobacter* sp. strain DZ-03-13-aga	99	+	+	+
LD20D12	*Pseudoalteromonas* sp. strain BSw22140	100	+	-	-

LD20E9	*Pseudoalteromonas* sp. strain BSw22134	99	+	+	+
LD20E11	*Pseudoalteromonas* sp. strain BSw22127	99	+	+	+
LD20F1	*Pseudoalteromonas translucida* strain BBCC2847	99	+	-	+
LD20F6	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	+
LD20F8	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	+
LD20F9	*Pseudoalteromonas* sp. strain BSw22136	100	+	-	+
LD20F12	*Pseudoalteromonas nigrifaciens* strain VSD915	100	+	-	
LD25A2	*Pseudoalteromonas nigrifaciens* strain BBCC2584	100	+	-	+
LD25B3	*Bacillus altitudinis* strain C341	100	+	+	-
LD25B7	*Bacillus pumilus* strain AN225_C1	100	+	-	-
LD25C1	*Bacillus circulans* strain PK3-138	99	-	+	-
LD25C2	*Pseudoalteromonas distincta* strain BBCC2856	100	+	-	+
LD25D2	*Bacillus altitudinis* strain C341	100	+	-	-
LD25D5	*Bacillus pumilus* isolate TD22	100	+	-	-
LD25D9	*Bacillus altitudinis* strain C341	100	+	+	-
LD25D10	*Pseudomonas* sp. strain WR1	99	+	+	-
LD25D11	*Bacillus altitudinis* strain C341	100	+	-	-
LD25E4	*Bacillus safensis* strain IAE257	99	+	-	-
LD25E6	*Bacillus safensis* strain IAE257	100	+	-	-
LD25E7	*Bacillus safensis* strain IAE257	100	+	-	-
LD25E9	*Bacillus safensis* strain IAE257	100	+	-	-
LD25E10	*Bacillus safensis* strain IAE257	99	+	-	-
LD25E11	*Bacillus safensis* strain IAE257	100	+	-	-
LD25E12	*Pseudomonas* sp. CC11I2	99	+	+	-
LD25F12	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	-
LD25G4	*Bacillus altitudinis* strain C341	100	+	-	-
LD25G7	*Bacillus aerius* strain UB02	100	+	+	-
LD25G8	*Pseudoalteromonas* sp. strain DZ-01-01-aga	100	-	-	-
LD25G10	*Stenotrophomonas* sp. strain GR27	100	-	+	-
LD25G11	*Pseudoalteromonas* sp. strain DZ-01-01-aga	100	+	-	-
LD25H7	*Bacillus altitudinis* strain C341	100	+	+	-
LD25H8	*Bacillus altitudinis* strain C341	100	-	+	-
LD25H11	*Stenotrophomonas* sp. DQ01	100	-	+	-
LD30A7	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	+
LD30B7	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	+
LD30B8	*Oceanobacillus* sp. strain TRB48	99	+	-	+
LD30C6	*Pseudoalteromonas translucida* strain BBCC2847	100	+	-	-
LD30C8	*Bacillus circulans* strain PK3-56	99	-	+	-
LD30D1	*Bacillus circulans* strain PK3-56	100	-	+	-
LD30D3	*Bacillus subtilis* strain isolate S331	100	+	+	-
LD30D5	*Bacillus subtilis* strain isolate S331	100	+	-	+

LD30D6	*Bacillus mojavensis* strain UQPM29	100	+	+	-
LD30E4	*Pseudoalteromonas translucida* strain BBCC2847	99	+	-	-
LD30E5	*Pseudoalteromonas* sp. strain BSw22136	100	+	-	+
LD30E8	*Oceanobacillus sojae* strain Y27	99	+	-	
LD30F8	*Oceanobacillus* sp. strain TRB48	99	+	-	+
LD30G6	*Bacillus subtilis* strain R37	100	+	+	+
LD30G7	*Pseudoalteromonas* sp. strain DZ-01-01-aga	100	-	-	+
LD30H5	*Pseudomonas helmanticensis* strain 4-3R	99	-	+	-
LD30H6	*Bacillus circulans* strain PK3-138	99	-	+	-
LD30H8	*Bacillus circulans* strain PK3-138	100	-	+	-
LD30H9	*Micrococcus aloeverae* strain Y23	100	-	+	-

7.3 Chapter 4

Table S4.1. Sampling metadata of *L. digitata*-associated bacterial communities

Sample ID	Sampling month	Sampling year	Algal part
APR-16-HF	APRIL	2016	HOLDFAST
APR-16-SP	APRIL	2016	STIPE
APR-16-MST	APRIL	2016	MERISTEM
APR-16-BD	APRIL	2016	BLADE
JULY-16-HF	JULY	2016	HOLDFAST
JULY-16-SP	JULY	2016	STIPE
JULY-16-MST	JULY	2016	MERISTEM
JULY-16-BD	JULY	2016	BLADE
NOV-16-HF	NOVEMBER	2016	HOLDFAST
NOV-16-SP	NOVEMBER	2016	STIPE
NOV-16-MST	NOVEMBER	2016	MERISTEM
NOV-16-BD	NOVEMBER	2016	BLADE
JAN-17-HF	JANUARY	2017	HOLDFAST
JAN-17-SP	JANUARY	2017	STIPE
JAN-17-MST	JANUARY	2017	MERISTEM
JAN-17-BD	JANUARY	2017	BLADE

Table S4.2. Results of the Kruskal-Wallis rank sum test and Dunn post-hoc test, testing for significant differences between the epibacterial communities associated with different parts of *Laminaria digitata* including holdfast (HF), stipes (SP), meristem (MST) and blades (BD).

Krsukal-Wallis chi-squared = 11.404, df = 3, p value = 0.009729

Comparison	**Z**	**P.unadj**	**P.adj**
BD - HF	1.4852213	0.137485208	0.206227811
BD - MST	-1.7080045	0.087635505	0.175271011
HF - MST	-3.1932258	0.001406929	0.008441572
BD - SP	-0.9653939	0.334347578	0.401217094
HF - SP	-2.4506152	0.014261233	0.042783700
MST - SP	0.7426107	0.457717434	0.457717434

Table S4.3. Results of the Kruskal-Wallis rank sum test and Dunn post-hoc test, testing for significant differences between the epibacterial communities associated with *Laminaria digitata* sampled at different months including April (APR), July (JULY), November (NOV) and January (JAN).

Krsukal-Wallis chi-squared = 1.8309, df = 3, p value = 0.6082

Comparison	**Z**	**P.unadj**	**P.adj**
APR - JAN	-0.5198275	0.6031838	0.7238206
APR - JULY	-1.3366992	0.1813208	1.0000000
JAN - JULY	-0.8168717	0.4140017	0.8280035
APR - NOV	-0.5198275	0.6031838	0.9047758
JAN - NOV	0.0000000	1.0000000	1.0000000
JULY - NOV	0.8168717	0.4140017	1.0000000